REMARKS

I. Provisional Obviousness-type Double Patenting Rejection

Claims 1-2, 8-9 and 15-16 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 6-9, 14-17 and 22-24 of copending application Serial No. 08/398,852. Applicant will submit a terminal disclaimer to overcome this rejection when the pending claims are indicated as being in condition for allowance.

II. Rejection under 35 U.S.C. § 112, first paragraph

Claims 1-4, 8-11, and 15-18 are rejected under 35 U.S.C. § 112, first paragraph, on the grounds that the specification is not enabling for effecting any changes in the biochemistry or function of the central nervous system. Applicant respectfully traverses this rejection.

Applicant's specification provides a description of how to treat diseases or disorders of the central nervous system with IGF-I or IGF-II. Several researchers have subsequently conducted research and published data that corresponding to that teaching, the results of which support the enabling character of applicant's disclosure. The following references, copies of which are enclosed with this response, disclose research results that demonstrate that the teachings of applicant's disclosure are indeed enabling:

Knusel et al., "Selective and Nonselective Stimulation of Central Cholinergic and Dopaminergic Development *in vitro* by Nerve Growth Factor, Basic Fibroblast Growth Factor, Epidermal Growth Factor, Insulin and the Insulin-like Growth Factors I and II," *J. Neurosci.* 10:

558-570 (1990), shows that IGF-I and IGF-II can support cultured septal cholinergic, pontine cholinergic and mesencephalic dopaminergic neurons in culture.

Liu and Lauder, "S-100ß and Insulin-Like Growth Factor-II Differentially Regulate Growth of Developing Serotonin and Dopamine Neurons In Vitro," *J. Neurosci. Res* 33:248-256 (1992), shows that IGF-II can stimulate neurite outgrowth in dopaminergic neurons from the substantial nigra/ventral tegmental area and cell body growth of serotoninergic rostral raphe as well as dopaminergic neurons in culture. This demonstrates that IGFs can treat or prevent neuronal damage to dopaminergic and serotoninergic neurons in the central nervous system.

Bozyczko-Coyne et al., "IGF-I Supports the Survival and/or Differentiation of Multiple Types of Central Nervous System Neurons," *Ann. NY Acad. Sci.* 692:311-313 (1993), shows that IGF-I can support the survival of cortical, basal forebrain, mesencephalic, retinal and spinal cord neurons in culture. Taken together with Knusel et al. (1990) and Liu and Lauder (1992), these references show that IGF-I and IGF-II can support a wide variety of brain and spinal cord neurons. The research on cholinergic and dopaminergic neurons is particularly significant as it is these neurons that are among those afflicted in Alzheimer's and Parkinson's Diseases.

Cheng and Mattson, "IGF-I and IGF-II Protect Cultured Hippocampal and Septal Neurons Against Calcium-mediated Hypoglycemic Damage," *J. Neurosci.* 12:1558-1566 (1992), shows that IGF-I and IGF-II can protect hippocampal and septal neurons from hypoglycemic damage in culture. The hippocampus is the area of brain involved in memory, and these data support applicant's earlier disclosure that IGFs can treat or prevent neuronal damage in diseases or disorders involving the hippocampus, such as Alzheimer's Disease or senile dementia.

Dore et al., "Insulin-like Growth Factor I Protects and Rescues Hippocampal Neurons Against ß-amyloid- and Human Amylin-induced Toxicity," *Proc. Natl. Acad. Sci USA* 94:4772-4777 (1997), shows that IGF-I and IGF-II can prevent toxicity of beta-amyloid and amylin in cultured hippocampal neurons. The beta-amyloid is a protein deposited in neuritic plaques and is believed to be pathologic in Alzheimer's Disease. This supports applicant's earlier disclosure that IGF-I and IGF-II can prevent or treat neuronal damage in Alzheimer's Disease.

Mozell and McMorris, "Insulin-like Growth Factor I Stimulates Oligodendrocyte Development and Myelination in Rat Brain Aggregate Cultures," *J. Neurosci. Res.* 30:382-390 (1991), shows that IGF-I stimulates myelination in cultures containing neurons and glia. This shows that applicant's earlier disclosure enables one to treat or prevent neuronal damage to neurons which may arise from demyelinating disorders or diseases, such as multiple sclerosis.

Carson et al., "Insulin-like Growth Factor I Increases Brain Growth and Central Nervous System Myelination in Transgenic Mice," *Neuron* 10:729-740 (1993), shows that brain size and myelination is increased in transgenic mice overexpressing IGF-I. There was a balanced growth, showing that all types of brain cells were increased. This supports applicant's earlier disclosure that IGFs have ubiquitous actions on all types of brain neurons and that IGFs can treat a wide variety of brain disorders and diseases.

Thornton et al., "Decreases in Type 1 Insulin-like Growth Factor Receptors in Cortex and Hippocampus of Aged Rats," *Soc. Neurosci. Abs.* 22:1234 (1996), shows that the type I IGF receptor is reduced in the hippocampus and cortex of aged rates. These regions of brain are involved in learning and memory.

Kitraki et al., "Aging-related Changes in IGF-II and C-FOS Gene Expression in the Rat Brain," *Int. J. Devl. Neurosci.* 11:1-9 (1993), shows that IGF-II gene expression is reduced with aging in rats. IGF-II is the predominant IGF in brain, and acts through the type I IGF receptor.

Mooney et al., "Insulin-like Growth Factor-1 (IGF-1) Increases Working Memory in Aged Animals," *Soc. Neurosci. Abs.* 20:192 (1994), shows that intracranial infusion of IGF-I can prevent loss of memory normally associated with aging in rats. Therefore, replacing the IGF lost with aging in brain may help preserve memory.

MacIntosh et al., "Efficacy of Insulin-like Growth Factor (IGF-1) in the Treatment of Behavioral and Cognitive Deficits Following Experimental Brain Injury," *Soc. Neurosci. Abs.* 20:192 (1994), shows that following concussive brain injury in rats there is loss of motor function, and such loss is prevented by post-injury administration of IGF-I. The blood-brain barrier is disrupted in this type of injury. These data show that IGF may be useful to treat or prevent neuronal damage following traumatic brain injury.

Saatman et al., "Insulin-like Growth Factor-1 (IGF-1) Improves both Neurological Motor and Cognitive Outcome Following Experimental Brain Injury," *Exptl. Neurol.* 147:418-427 (1997), shows that following fluid concussive brain injury, where a large number of different types of neurons can be injured, IGF-I was administered parenterally. Parenteral IGF-I treatment was shown to reduce motor dysfunction and memory loss in injured rats.

Hatton et al., "Intravenous Insulin-like Growth Factor-I (IGF-I) in Moderate-to-severe Head Injury: A Phase II Safety and Efficacy Trial," *J. Neurosurg.* 86:779-786 (1997), shows that the parenteral administration of IGF-I can improve the clinical outcome of patients with

moderate-to-severe traumatic brain injury. This reference directly supports applicant's earlier disclosure that parenterally administered IGFs can treat or prevent neuronal damage in the brain.

Applicant therefore submits that parenteral treatment with IGF-I or IGF-II is applicable to a variety of central nervous system diseases and disorders, as confirmed by the scientific literature. Therefore, applicant requests that the rejection of claims 1-4, 8-11, and 15-18 under 35 U.S.C. § 112, first paragraph, be withdrawn.

III. Rejection under 35 U.S.C. § 112, second paragraph

Claims 1-4, 8-11 and 19-20 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as his invention. Applicant respectfully traverses this rejection.

The rejection of claims 1-4 and 8-11 appears to be due to uncertainty to the Examiner regarding the meaning of the phrase "change in the biochemistry or function of the central nervous system." The claims are amended above to recite the claimed method in terms of methods for "treating or preventing neuronal damage".

The Examiner also objects to the language "an amount effective to treat" in claims 19 and 20. These claims are also amended above to clarify that the amount is effective to treat "a noradrenergic neuron affected by a disease or disorder of the central nervous systems".

Applicant respectfully requests that the rejection of claims 1-4, 8-11, and 19-20 under 35 U.S.C. § 112, second paragraph, be withdrawn.

IV. Rejection under 35 U.S.C. § 102(e)

Claims 1-6, 8-13, and 15-18 are rejected under 35 U.S.C. §102(e) as being anticipated by Lewis et al. Applicant respectfully traverses this rejection.

The Examiner states that "Lewis et al. teach all the limitations of the claims, and without specific evidence to the contrary Lewis et al. teachings are enabling." Applicant contends, however, that the Examiner has not yet established a case of enablement. The Examiner has not shown that Lewis et al. even recognized the potential for treatments of the central nervous system with IGF-I or IGF-II across the blood-brain barrier. Instead, Lewis et al. teaches away from such a possibility by characterizing the blood-brain barrier as a problem:

> Where the polypeptide is intended for use as a therapeutic for disorders of the CNS, an additional problem must be addressed: overcoming the so-called "blood-brain barrier," the brain capillary wall structure that effectively screens out all but selected categories of molecules present in the blood, preventing their passage into the brain. While the blood-brain barrier may be effectively bypassed by direct infusion of the polypeptide into the brain, the search for a more practical method has focused on enhancing transport of the polypeptide of interest across the blood-brain barrier, such as by making the polypeptide more lipophilic, by conjugating the polypeptide of interest to a molecule which is naturally transported across the barrier, or by reducing the overall length of the polypeptide chain.

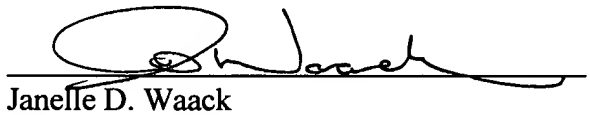
Lewis et al., col. 3, ll. 44-59. Lewis et al. then describes various means to get an IGF molecule across the blood-brain barrier, which means are limited to modifying the IGF molecule, *e.g.*, by chemical modification such as conjugation to cationized albumin (col. 6, ll. 50-55), substituting or deleting certain amino acids (col. 7, l. 52 - col. 8, l. 29), or by intracerebral injection (Example 4). Lewis et al. simply does not state that the IGF-I or IGF-II molecule alone can be an effective method for treating a disease or disorder of the central nervous system. As such, it cannot be a

basis for rejecting applicant's claims defining such treatment of the central nervous system by parenteral administration of IGF-I or IGF-II.

V. Conclusion

In conclusion, in view of the foregoing amendment and remarks, applicant respectfully submits that all outstanding rejections are overcome and believes this case to be in condition for allowance. If the Examiner has any questions or comments regarding this application, he is invited to telephone the undersigned assignee's representative.

Respectfully submitted,



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Intravenous insulin-like growth factor-I (IGF-I) in moderate-to-severe head injury: a Phase II safety and efficacy trial

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The purpose of this study was to determine the effect of insulin-like growth factor-I (IGF-I) on the catabolic state and clinical outcome of head-injured patients. Thirty-three patients between the ages of 18 and 59 years with isolated traumatic head injury and Glasgow Coma Scale (GCS) scores of 4 to 10 were randomized to one of two groups. All patients received standard neurosurgical intensive care plus aggressive nutritional support; the patients in the treatment group also received intravenous therapy with continuous IGF-I (0.01 mg/kg/hour).

During the 14-day dosing period, the control patients lost weight, whereas treated patients gained weight despite a significantly higher measured energy expenditure and lower caloric intake ($p = 0.02$). Daily glucose concentrations and nitrogen outputs were greater in control patients ($p = 0.03$) throughout the study period. During Week 1, only treated patients achieved positive nitrogen balance. Fifteen of 17 treated and 13 of 16 control patients survived the 1st week. No deaths occurred in patients whose serum IGF-I concentrations were higher than 350 ng/ml. Dichotomized Glasgow Outcome Scale scores for patients with baseline GCS scores of 5 to 7 improved from poor to good for eight of 12 treated patients but for only three of 11 control patients ($p = 0.06$). Eight of 11 treated patients with serum IGF-I concentrations that were at least 350 ng/ml achieved moderate-to-good outcome scores at 6 months, compared to only one of five patients with lower concentrations ($p < 0.05$). These findings indicate that pharmacological concentrations of IGF-I may improve clinical outcome and nitrogen utilization in patients with moderate-to-severe head injury.

KEY WORDS • **brain injury** • **insulin-like growth factor-I** • **nitrogen balance** • **Glasgow Coma Scale** • **nutritional support** • **nutrition** • **treatment outcome**

PATIENTS suffering moderate-to-severe traumatic brain injury (TBI) demonstrate both hypermetabolism (increased energy expenditure) and hypercatabolism (increased protein loss) for up to several months after the insult.[14,24,47,63] Many investigators have found it difficult to achieve either positive caloric balance or positive nitrogen balance despite aggressive nutritional support.[14,24,47,63] When nutritional support is initiated early with aggressive administration of nonprotein calories, caloric balance is possible, but high nitrogen excretion rates and negative nitrogen balance persist.[6,13] The acute-phase response and hyperdynamic condition after head injury cannot be overcome with nutritional support alone.[14,43–45,61] The clinical consequences of inefficient nutrition after acute head injury include impaired immune function, delay in wound healing, muscle loss, and death.[51,52]

Pharmacological strategies to improve the use of nutrients during acute injury are under investigation.[21,35,50,59,60,66] Results with growth hormone have been inconsistent, depending on the severity of the injury and the timing of administration.[4,21,35,50,55,60,66] Insulin-like growth factor-I (IGF-I) is believed to mediate many of the anabolic effects of growth hormone.[10,31] Endogenous concentrations of IGF-I are depressed after brain injury.[27] Severe stress may induce a state of growth hormone resistance, leading to diminished synthesis of IGF-I in response to growth hormone; however, this finding has not been observed by all investigators.[4,16,33,55]

Insulin-like growth factor-I is a neurotrophic factor and has neuroprotective effects.[9,18,42,56] Higher endogenous concentrations of IGF-I are associated with improved survival after TBI.[27] Restoring IGF-I concentrations by administering recombinant human IGF-I (rhIGF-I) after TBI may benefit the patient's neurological response and may also have an effect on the patient's nitrogen balance. The purpose of this investigation was to determine the effect of rhIGF-I on the catabolic state and clinical outcome of patients who have suffered head injury.

Clinical Material and Methods

Study Design and Patient Population

This Phase II, open-label, prospective randomized trial was conducted at the University of Kentucky and the University of Tennessee at Memphis. The open-label design was requested by the United States Food and Drug Administration because IGF-I had not previously been administered to patients with TBI. Consecutively admitted patients between the ages of 18 and 59 years who had suffered TBI and had Glasgow Coma Scale[57] (GCS) scores of 4 to 10 were eligible. Patients were excluded for clinically significant disease or trauma other than head injury, chronic inflammatory diseases, spinal cord injury, diabetes, cancer, or uncontrolled seizures. Those treated by administration of more than 20 mg dexamethasone or its equivalent before randomization, those for whom death appeared to be imminent, and pregnant women were also excluded. After informed consent had been obtained, the patients were randomized on the basis of peak 6-hour postinjury GCS score, steroid dose, and age. All patients received standard neurosurgical intensive care combined with aggressive nutritional support as previously described;[64] the treatment group also received therapy with IGF-I. Intracranial pressure monitors were placed in all patients with GCS scores less than 8, according to routine neurotrauma care. This protocol was approved by the institutional review boards of both universities.

Drug Administration/Monitoring

Insulin-like growth factor-I was provided (Genentech, San Francisco, CA) as a sterile solution containing 5 mg rhIGF-I/1 ml citrate/NaCl, pH 6. The IGF-I was administered by peripheral continuous intravenous infusion at 0.01 mg/kg/hour beginning within 72 hours of injury and continuing for up to 14 days. During the 1st study day, pharmacokinetic sampling of blood was performed at baseline; at 5, 10, 15, 20, 30, 45, 60, and 90 minutes; and at 2, 3, 4, 6, 12, and 24 hours after initiation of the infusion. Serum concentrations of IGF-I were determined every 24 hours on Days 2 through 13. After the drug was discontinued, blood samples were collected at 0, 5, 10, 15, 20, 30, 45, and 60 minutes and at 2, 2.5, 3, and 4 hours for IGF-I quantification. Samples for determining serum concentrations of IGF-I and binding proteins were collected from control patients every morning. All assays were performed by Genentech.[38] Hematology, blood chemistry, urinalysis, creatinine clearance, partial thromboplastin time, and levels of prothrombin, free thyroxine (T_4), and thyroid-stimulating hormone were assessed regularly throughout the 14-day treatment period and at Day 28.

Nutritional Support

The goal of nutritional support was to provide nonprotein calories equal to 1.25 times the measured energy expenditure (MEE) plus 2 g protein/kg/day for both groups. The MEE was determined by indirect calorimetry. Nonprotein calories were adjusted if the MEE changed by more than 500 calories. The route of nutritional support was not dictated by the protocol. Parenteral nutrition was used until bowel sounds returned and gastric residual volumes were less than 700 ml in 24 hours. Lipid (Intralipid, 20%) provided approximately 30% of nonprotein calories during administration of parenteral nutrition.

Energy expenditure in all patients was measured by indirect calorimetry at baseline and on study Days 2, 3, 4, 6, 8, 10, 12, and 15. Twenty-four hour urine collections were obtained daily through Day 15. Urinary concentrations of nitrogen and 3-methylhistidine (3MH) were analyzed according to their chemiluminescence. Patients were weighed on Days 1, 4, 7, 10, 13, and 15 and at follow-up periods. Anthropometric measurements (triceps skinfold and mid-arm circumference) were recorded on the same schedule as weight.

For patients receiving IGF-I, serum glucose was measured on Day 1 with each pharmacokinetic sample. After the 1st day, serum glucose was monitored by either finger stick or serum concentration every 6 hours. For the control group of patients, glucose monitoring was performed every 12 hours. Serum glucose concentrations were analyzed by using the hexokinase method. Insulin was administered according to a sliding scale protocol when glucose concentrations were greater than 200 mg/dl. Hypoglycemia was defined as a serum glucose concentration of 70 mg/dl or less. Concentrations of C-peptide and insulin concentrations were measured by radioimmunoassay on Days 1 through 15 and on Day 28.

Clinical Outcome

Both GCS scores and intracranial pressures were recorded daily throughout the dosing period. All patients underwent follow-up examinations to assess nutritional and neurological outcome at Day 15, Day 30, the time of hospital discharge, and 3 months and 6 months after injury. Assessments of anthropometrics, weight, GCS score, and Glasgow Outcome Scale[34] (GOS) score were continued throughout this follow-up period.

Statistical Analysis

The metabolic endpoints examined during the study were MEE, nitrogen balance, serum glucose concentration, and urinary excretion of 3MH. These variables were compared between groups by using repeated-measures analysis of covariance with age, GCS score, and prerandomization administration of steroids as covariates. The clinical endpoints were weight, anthropometrics, GCS score, and GOS score at Day 15, Day 28, and 3 months and 6 months postinjury. A linear mixed-effects model for an unbalanced repeated-measures design was used to compare mean responses between groups for these endpoints.

Calorie and nitrogen variables, laboratory changes, and IGF-I concentrations were compared by using paired and two-sample Student's t-tests. Analysis of documented infections and outcome scores was based on Fisher's exact test for sparse contingency tables and chi-square statistics. Statistical significance was set at $p < 0.05$.

Results

Patient Demographics

At the two sites, a total of 33 patients were randomized. There were no significant differences in demographic data

TABLE 1

Clinical characteristics of 33 head-injured patients

Factor	Control Group (16 patients)	rhIGF-I–Treated Group (17 patients)
gender (no. of patients)		
female	2	3
male	14	14
age (yrs)		
mean (SD)	27.8 (9.21)	27.6 (7.7)
median	24	27
range	18–46	18–40
weight at baseline examination (kg)		
mean (SD)	75.7 (14.89)	71.3 (12.22)
median	71.1	68.5
range	61–115	53–100
baseline GCS score*		
mean (SD)	6.1 (2.16)	7 (2.25)
median	6	7
range	3–10	4–12
type of head injury (no. of patients)		
closed head injury	8	11
skull fracture	4	2
subdural hematoma	2	0
epidural hematoma	3	2
intracerebral hematoma	1	1
intracranial missile	1	2
elapsed time to treatment (hrs)		
mean (SD)	57.1 (8.52)	56.5 (9.91)
median	54.5	54
range	41–73	44–73
time on ventilator (days)		
mean (SD)	11.4 (5.29)	11.2 (5.86)
median	14.5	16
range	3–16	3–16

* Obtained immediately before administration of first dose.

between the groups (Table 1). Steroids (< 20 mg dexamethasone equivalent) were administered to two control patients and to one treated patient before randomization. One patient in the treatment group received 100 mg methylprednisolone as a single dose on study Day 1 for the management of optic neuritis. Fourteen of 17 patients treated with IGF-I completed the 14-day course of therapy. Thirteen of the 16 control patients completed 14 days of the study. Two treated patients and three control patients died during the 14-day dosing period. Therapy was discontinued for a patient in the treatment group because of tachycardia accompanied by persistent agitation. These symptoms continued after discontinuation of the study drug and were not attributed to the IGF-I therapy. All patients received parenteral nutrition as the initial route for nutrition support. Transition to enteral nutrition was accomplished during the 14-day study course for 14 control patients and 13 IGF-I–treated patients. The length of parenteral nutrition compared to enteral nutrition was not different between the groups; most patients received a combination of each as the transition process was applied. The average length of hospital stay was 42 days for survivors in both groups.

Clinical events are summarized in Table 2. No serious life-threatening event was attributed to IGF-I treatment. Patients received standard care for neurosurgical intensive care patients, including courses of antibiotic medications, anticonvulsant agents when required, mannitol, diuretics,

TABLE 2

*Clinical events in 33 head-injured patients**

Factor	Control Group (16 patients)	rhIGF-I–Treated Group (17 patients)
ICP monitoring (no. of patients)	12	14
peak ICP values (mm Hg)	25 ± 8	21 ± 3.5
infections (no. of patients)	14	12
episodes of pneumonia	10	11
episodes of bacteremia	8	1
episodes of urinary tract infection	5	1
episodes of meningitis	1	1
decubitus ulcers	3	0
cellulitis	0	1
diarrhea	8	3
diabetes insipidus	1	3

* ICP = intracranial pressure.

and histamine antagonists. No treatment differences were noted between the groups. No differences in vital signs, hematological analyses, or blood chemistry were noted between the groups. Pretreatment free T_4 values were within normal limits in both groups. Total pretreatment T_4 values were reduced in six of 16 control patients and in nine of 17 IGF-I–treated patients. Pretreatment thyroid-stimulating hormone values were reduced in three treated patients and four control patients. These values returned to normal limits at Day 15, with the exception of total T_4 in one IGF-I–treated patient.

Nutritional Outcome

Energy Expenditure. Baseline MEEs were 2381 ± 369 kcal/day (mean ± standard deviation [SD]) in control patients and 2562 ± 586 kcal/day in the treated group. These baseline energy expenditures were from 1 to 1.6 times the predicted energy expenditure in control patients and from 1.1 to 2 times that in treated patients.[32] Overall, control patients had a lower MEE ($p = 0.01$) and higher caloric intake ($p = 0.02$) than patients treated with IGF-I (Fig. 1). The overall average caloric intake was 40 kcal/kg/day in control patients and 36 kcal/kg/day in treated patients. Caloric balance (nonprotein intake minus MEE) was significantly different between the groups (Fig. 1). During both weeks, the control patients had a more positive caloric balance than the treated patients ($p = 0.04$).

Nitrogen. The overall mean protein intake was 1.8 ± 0.14 g/kg/day in control patients and 1.6 ± 0.35 g/kg/day in treated patients. Nitrogen intake during Week 1 was not significantly different between the groups; however, during Week 2, intake in the treated group was significantly less than that in the control group ($p = 0.002$) (Fig. 2). Nitrogen output in the control group was greater than that of the treated group during both study weeks ($p = 0.04$). The treated group achieved a positive mean nitrogen balance during Week 1 (Fig. 3), but the control group did not. Although nitrogen output was lower in treated patients during Week 2, nitrogen balance was negative in both groups during Week 2. There was no significant difference in 3MH excretion between groups.

Weight. Despite improved caloric balance and higher

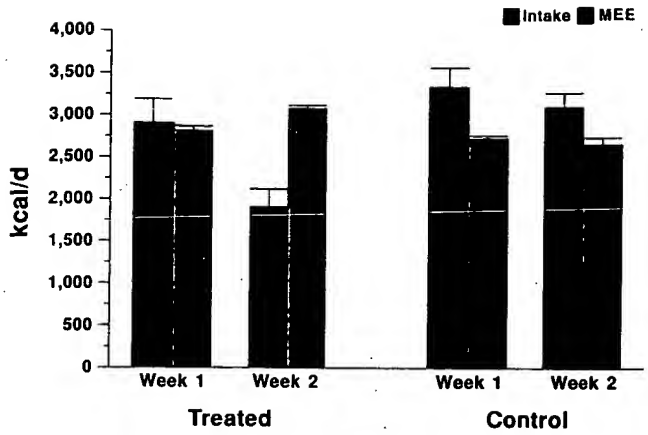

FIG. 1. Bar graph displaying calorie intake and MEE in control and IGF-I–treated patients. Nonprotein calorie intake was greater in the control group (3260 ± 229 kcal/day [d]) than in the group treated with IGF-I (2576 ± 560 kcal/d; p = 0.02). Measured energy expenditure was higher in the IGF-I group (2908 ± 149 kcal/d) than in the control group (2700 ± 52 kcal/d; p = 0.01).

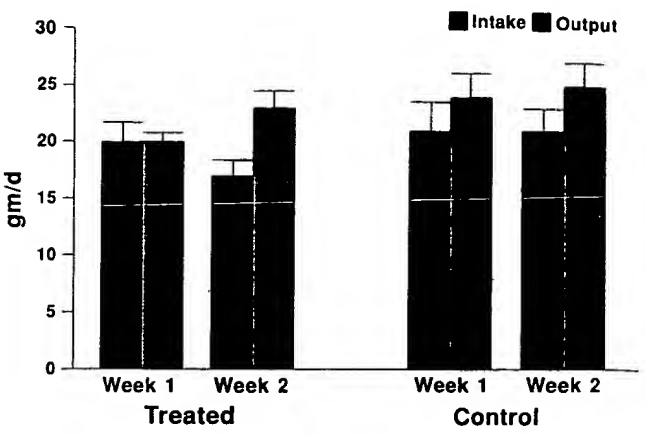

FIG. 2. Bar graph showing nitrogen input and output in head-injured patients. Nitrogen intake was not different between the two groups during Week 1 (control group 21 ± 2.6 g/day [gm/d] vs. treated group 20 ± 1.7 gm/d). During Week 2, nitrogen intake was significantly lower in the IGF-I–treated group (control group 21 ± 2 gm/d vs. treated group 17.3 ± 1.4 gm/d; p = 0.002). Nitrogen output was higher in control patients in Week 1 (control group 25 ± 2.1 gm/d vs. treated group 20 ± 0.8 gm/d; p = 0.004). During Week 2, nitrogen output increased in the IGF-I–treated group but remained lower than that in the control patients (control group 25 ± 2.3 gm/d vs. treated group 23 ± 1.5 gm/d; p = 0.04).

nitrogen intake, the weight of control patients had decreased by 2.8 ± 11.1% at Day 15. Conversely, at the same time point, the weight of treated patients had increased by 2.3 ± 8.9% above admission weights. Anthropometric values did not differ significantly between the groups.

Glucose. Average daily glucose concentrations were higher among control patients (179 ± 21 mg/dl) than among treated patients (146 ± 12 mg/dl) throughout the protocol (p = 0.03). One patient in the control group required treatment for a glucose concentration of 63 mg/dl on Day 13 during transition to enteral feeding. Similarly, two IGF-I–treated patients received a dextrose bolus on Day 13 while receiving enteral nutrition. Patients had no symptoms of hypoglycemia; low blood sugar concentrations were identified by protocol-driven glucose monitoring. Serum insulin concentrations were elevated in both groups. There was no difference in insulin dose or in the number of patients treated with insulin between the groups. The C-peptide values were increased for all patients in both groups, but the differences were not statistically significant.

Serum IGF-I Concentrations

At baseline, endogenous serum IGF-I concentrations were below reported physiological concentrations (150–400 ng/ml) for all patients (Fig. 4).[31] Concentrations of IGF-I above 150 ng/ml were achieved for all treated patients but for only six control patients ($p < 0.01$). Physiological concentrations were restored for all treated patients within the first 24 hours of dosing. The average time for restoration of physiological levels in the control group was significantly delayed to 7 ± 4.5 days ($p < 0.01$).

The average IGF-I concentrations at Week 1 and Week 2 in the control group were significantly lower than that of the IGF-I–treated group (Fig. 4; p = 0.0001 at Week 1; p = 0.02 at Week 2). The average peak concentration for IGF-I–treated patients was 458 ± 178 ng/ml, compared with 156 ± 99.7 ng/ml for control patients ($p < 0.01$). The time to peak concentration was 4 ± 2.3 days for treated patients, compared with 11 ± 4.5 days for control patients ($p < 0.01$). For 13 IGF-I–treated patients, IGF binding protein (BP)-3 concentrations decreased from 2.8 ± 0.2 to 2.1 ± 0.2 mg/dl. Concentrations of IGFBP-2 increased from 275 ± 46 μg/ml to 678 ± 145 μg/ml over the 14-day period. Plasma growth hormone concentrations declined from 2.3 ± 0.6 μg/L to 0.4 ± 0.1 μg/L.

Serum IGF-I concentrations of 350 ng/ml were reached for 11 treated patients during the study; for eight of these patients, this level was achieved during the first 24 hours of infusion. Despite continuous drug administration, serum IGF-I concentrations greater than 350 ng/ml were maintained only for an average of 7.5 ± 3.8 days. For six treated patients, serum IGF-I concentrations did not reach peaks greater than 350 ng/ml at any point in the dosing period; two of these treated patients and three control patients died within the 1st week of injury. The baseline predose concentration of IGF-I was higher for patients whose serum IGF-I concentrations were above 350 ng/ml during the infusion (80 ± 50 ng/ml vs. 40 ± 8.9 ng/ml, $p < 0.05$).

Neurological Outcome

After randomization, the 6-hour postinjury score of one patient had improved to 12 before dosing; data from this patient are not included in the neurological outcome analysis. Three deaths occurred in the treated group and five in the control group. Within the 1st week, two patients in the IGF-I–treated group and three in the control group died. Early deaths were due to severe brain injury in three cases, sepsis in one case, and multiple organ dysfunction

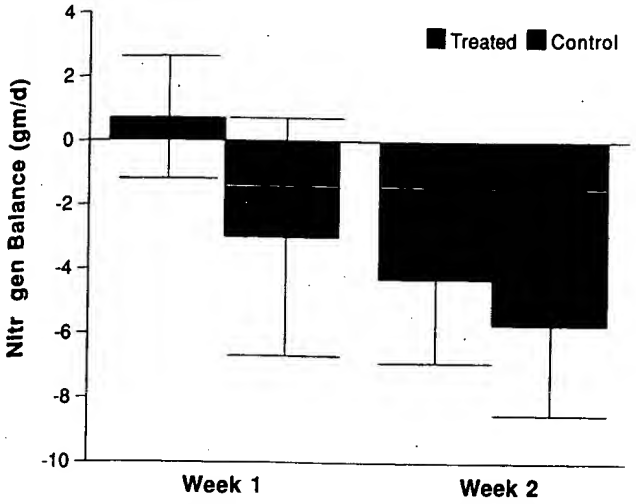

FIG. 3. Bar graph showing nitrogen balance in head-injured patients. Nitrogen balance was significantly more positive in the IGF-I–treated group during Week 1 (treated group 0.7 ± 1.9 gm/d vs. control group −3 ± 3.7 gm/d; p = 0.04). During Week 2, nitrogen balance was not significantly different (control group −5.7 ± 2.8 gm/d vs. treated group −4.3 ± 2.6 gm/d).

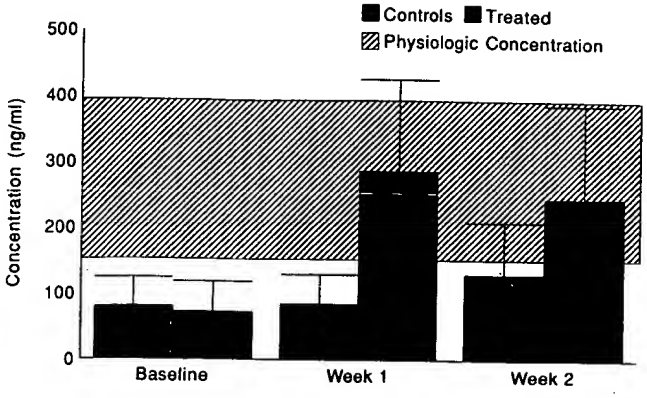

FIG. 4. Bar graph showing serum concentrations of IGF-I from baseline through the second week of treatment. Serum concentrations of IGF-I were depressed below 150 ng/ml after TBI. Administration of IGF-I restored physiological concentrations within 24 hours of dosing. The average concentration of IGF-I in Week 1 was 85 ± 45 ng/ml in the control group and 288 ± 138.5 ng/ml in the treated group (p = 0.001). During the 2nd week after injury, the control patients had an average serum concentration of 130 ± 79.5 ng/ml, compared with 246 ± 143 ng/ml in the treated group (p = 0.02).

syndrome in one other case. A favorable outcome trend was observed for treated patients surviving the 1st week after injury with GCS scores of 5 to 7 (Fig. 5). Dichotomized GOS scores improved from poor to good for eight (67%) of 12 treated patients, compared with three (27%) of 11 control patients (p = 0.06)

The effect of serum IGF-I concentration on the outcome of patients was evaluated. Baseline IGF-I concentrations were not significantly different between survivors (84 ± 48 ng/ml) and nonsurvivors (55 ± 32 ng/ml). No deaths were reported among the 11 patients who achieved serum IGF-I concentrations greater than 350 ng/ml, compared with three deaths among the five patients who did not achieve this concentration (p = 0.02). In the treated group, eight of 11 patients with serum IGF-I concentrations greater than 350 ng/ml achieved moderate-to-good outcome scores at 6 months, compared with only one of five patients with lower concentrations ($p < 0.05$).

Discussion

Head-injured patients treated with IGF-I gained weight and retained nitrogen despite a negative caloric balance and lower nitrogen intake. Previous investigations have demonstrated hypercatabolism and protein loss to be unresponsive despite protein replacement at doses up to 2.5 g/kg/day for severely stressed patients.[6,13,63] Low IGF-I concentrations may contribute to the inefficient use of exogenous protein after head injury.[12,46] All head-injured patients had depressed baseline IGF-I concentrations, and these depressed concentrations persisted for approximately 7 days in the control group. In contrast, administration of IGF-I restored serum concentrations to at least 150 ng/ml for all treated patients within the first 24 hours of dosing. Improved nitrogen balance occurred when IGF-I concentrations were restored to the physiological range. In the current study, the control patients had greater nitrogen output over the study period than the treated patients, despite higher protein intake. Nitrogen retention appeared to be more efficient in the treatment group. The poor caloric intake may have limited the efficacy of IGF-I during the 2nd week; however, low nitrogen intake makes it difficult to interpret this finding conclusively.[12,46]

Nutritional support has not been effective in reducing nitrogen excretion after TBI.[6,58,64] Therapeutic strategies for overriding the catabolic state have been tried in a variety of populations.[21,35,50,59,60,66] Growth hormone has successfully reduced urinary nitrogen loss in both surgically treated and burn populations.[22,35,50,60,66] This effect of growth hormone was not observed after multiple trauma.[4,55] The variability in growth hormone response after TBI, combined with the side effects of hyperglycemia and fluid retention, may limit its potential for use in traumatic brain injury.[10,37]

Insulin-like growth factor-I mediates many of the anabolic effects of growth hormone.[22,31] Within the liver, the binding of growth hormone to receptors initiates the production of IGF-I and its associated binding proteins.[31] This process is dependent on adequate nutritional intake.[31,46] The IGF-I acts both systemically and locally to suppress protein degradation, increase amino acid uptake, and increase cellular proliferation and differentiation.[22] Severe stress and acute disease alter normal patterns of increased IGF-I in response to nutrients.[8,33] When serum concentrations were increased by administration of systemic IGF-I therapy, protein oxidation was reduced after acute thermal injury.[11] Similarly, when physiological concentrations of IGF-I were restored in our head-injured patients receiving IGF-I, nitrogen excretion was lower than that in the control group. The association of reduced nitrogen excretion with IGF-I therapy deserves additional research.

The hypoglycemic effect of IGF-I may be particularly attractive during administration of specialized nutritional

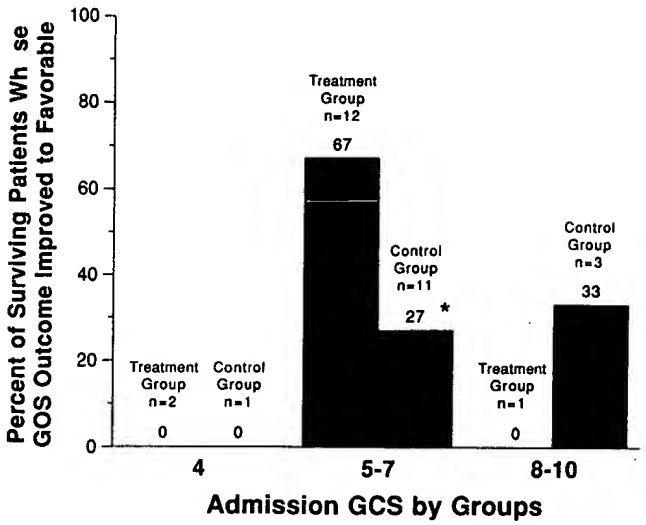

FIG. 5. Bar graph showing patient outcome as it relates to admission GCS score. All surviving patients admitted with a GCS score of 4 remained vegetative or severely disabled (according to GOS scores). For eight patients in the treated group and three patients in the control group admitted with GCS scores of 5 through 7, GOS scores improved from poor outcome at Day 15 to moderate disability or good recovery ($p = 0.06$). Patients with admission GCS scores of 8 through 10 had 15-day GOS scores of good, except for one of the control patients whose GOS score improved from vegetative to good recovery at 6 months.

support. Previous investigations of TBI have demonstrated higher mortality rates when patients were significantly underfed.[52] Improved neurological outcome was seen when adequate nutritional support was implemented early.[64] Although nutrition protocols were standardized and initiated early in our study, nutrient intake was lower in the treated group. Despite the compromise in intake, the positive trends continued in the treatment group.

The glucose-modulating properties of IGF-I may contribute to its potential beneficial effect in the treatment of patients with TBI. Hyperglycemia is common after TBI and is associated with poor outcome.[40,54,62] The hypoglycemic potential of IGF-I could contribute to a neurological benefit for patients with TBI.[29] Serum glucose concentrations were significantly lower among our patients treated with IGF-I. This clinical response is similar to observations among other IGF-I–treated populations, but the difference in caloric intake may also have contributed to this observation.

Concentrations of IGF-I may influence mortality after TBI.[27] Gottardis, et al.,[27] observed that patients with TBI who died had lower IGF-I concentrations than survivors. In our study, admission IGF-I concentrations did not predict survival for our patients. However, rapid restoration of physiological IGF-I concentrations for the treated group may explain the different clinical outcomes. The significant difference in the number of deaths between treated patients with IGF-I concentrations above 350 ng/ml and those with lower concentrations suggests that more definitive studies are needed.

Treated patients with admission GCS[57] scores of 5 to 7 who survived the initial week after injury had improved neurological outcomes compared with similar patients in the control group. In the treated group, patients with serum concentrations exceeding 350 ng/ml during the dosing phase had a greater improvement in neurological outcome scores at 6 months. The concentration of 350 ng/ml is near the upper limit of reported normal values and may indicate a minimum concentration for therapeutic efficacy. The full extent of the effects of IGF-I at higher concentrations for extended periods is not known.

Despite continuous infusion, serum concentrations greater than 350 ng/ml could not be maintained throughout the 14-day dosing period. A change in IGF-I clearance would be consistent with the negative feedback of IGF-I on growth hormone secretion.[5] A decrease in growth hormone alters the production of IGFBP-3, the principal binding protein that prolongs the systemic half-life of IGF-I.[3,5,7] A lower concentration of IGFBP-3, as a result of lower production of endogenous growth hormone, reduces the half-life of IGF-I from approximately 18 hours to only 8 minutes.[30] We measured IGFBP-3, IGFBP-2, and growth hormone in 13 of our IGF-I–treated patients. A decline in IGFBP-3 was observed and found to be associated with falling IGF-I concentrations and rising IGFBP-2 concentrations.[7] Growth hormone concentrations also decreased during IGF-I treatment.[7] Dosing strategies to override this effect will be needed if the relationship of IGF-I concentrations and neurological improvement is to be more definitively evaluated.

Higher serum concentrations may improve bioavailability to the injured brain. Insulin-like growth factor-I receptors have been found throughout the brain and persist throughout the aging process.[1,2,15,17] Separate receptors for IGF-I have been isolated on brain capillaries.[23] The maximum binding of the IGF receptor is approximately fivefold greater than that for the blood-brain barrier insulin receptor.[49] The IGF-I is internalized by isolated brain capillaries, suggesting the presence of a transport system. These findings lead one to speculate that circulating IGF-I may be transported into the brain via these existing systems.

Insulin-like growth factor-I is a growth factor present within the central nervous system.[53] Both neurological and neuroprotective properties have been attributed to IGF-I.[9,18,28,42,56] An increased expression of IGF-I within the central nervous system has been demonstrated after brain injury.[25] Intraventricular administration of IGF-I in a hypoxic–ischemic animal model reduced central nervous system neuronal loss by more than 80%.[26] Therefore, several factors could be responsible for neurological improvement after the administration of IGF-I.

Conclusions

Several promising investigational therapies have failed to produce improved outcome once tested in large, multicenter trials.[19,20,36,41,48,65] Additional research will be needed to evaluate the potential of IGF-I as a therapeutic agent for patients with head injury. In this small pilot study, significant trends in improved outcome and nutritional response were observed despite the limitation in population size and difference in nutrient intakes. Modified dosing strategies should be tested for a larger population of patients

with TBI to clarify the potential of IGF-I for improving neurological outcome. Evidence from normal volunteers and cachectic patients with acquired immune deficiency indicates that growth hormone–dependent binding proteins need to be restored along with IGF-I to optimize serum concentrations and promote anabolism.[36,39] Defining optimal serum and cerebrospinal fluid concentrations and determining a dosing strategy for maintaining those concentrations will be the goals of further investigations using this therapy.

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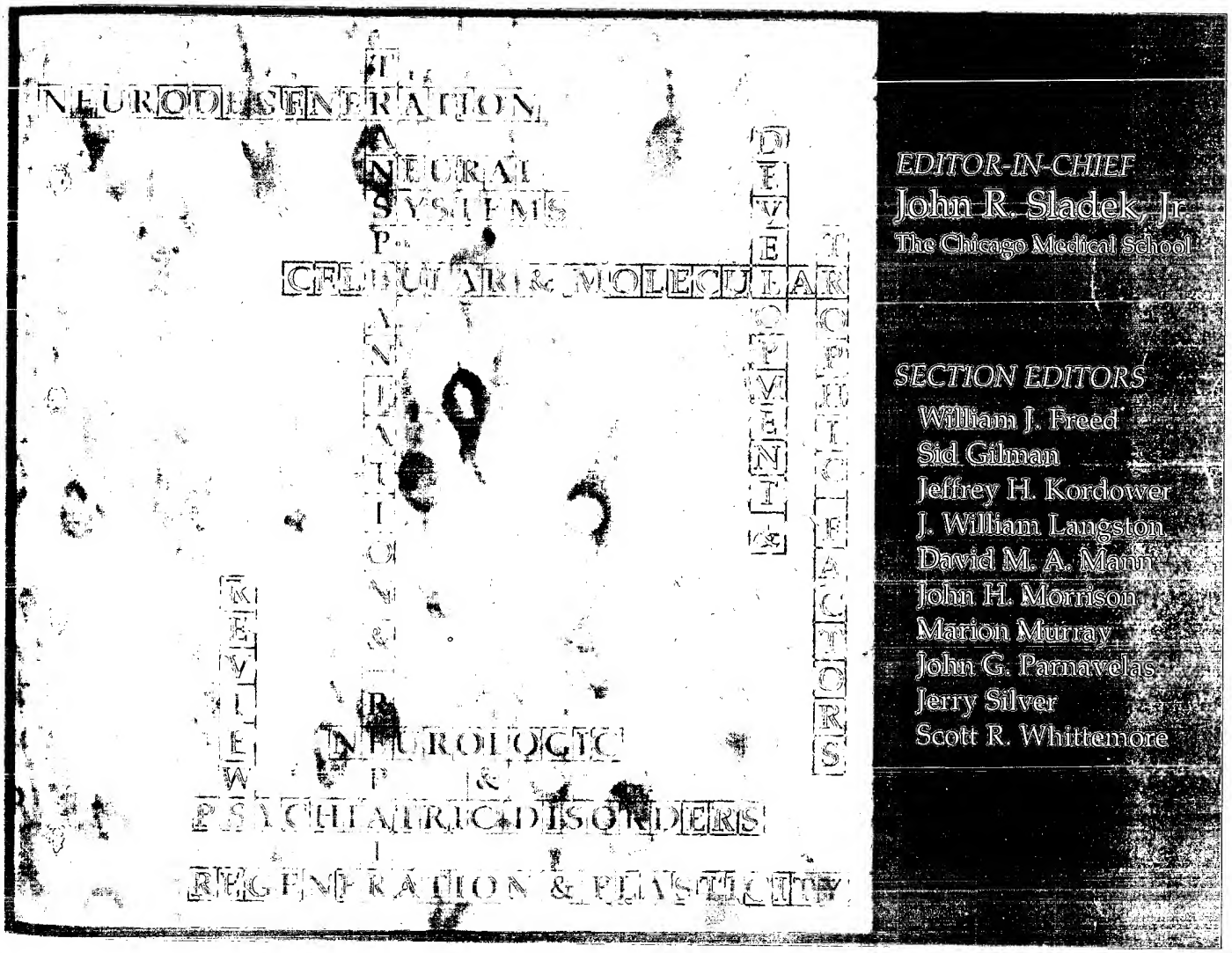

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Insulin-like Growth Factor-1 (IGF-1) Improves both Neurological Motor and Cognitive Outcome Following Experimental Brain Injury

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We evaluated the efficacy of insulin-like growth factor-1 (IGF-1) in attenuating neurobehavioral deficits following lateral fluid percussion (FP) brain injury. Male Sprague–Dawley rats (345–425 g, $n = 88$) were anesthetized and subjected to FP brain injury of moderate severity (2.4–2.9 atm). In Study 1, IGF-1 (1.0 mg/kg, $n = 9$) or vehicle ($n = 14$) was administered by subcutaneous injection at 15 min postinjury and similarly at 12-h intervals for 14 days. In animals evaluated daily for 14 days, IGF-1 treatment attenuated motor dysfunction over the 2-week period ($P < 0.02$). In Study 2, IGF-1 (4 mg/kg/day, $n = 8$ uninjured, $n = 13$ injured) or vehicle ($n = 8$ uninjured, $n = 13$ injured) was administered for 2 weeks via a subcutaneous pump implanted 15 min postinjury. IGF-1 administration was associated with increased body weight and mild, transient hypoglycemia which was more pronounced in brain-injured animals. At 2 weeks postinjury ($P < 0.05$), but not at 48 h or 1 week, brain-injured animals receiving IGF-1 showed improved neuromotor function compared with those receiving vehicle. IGF-1 administration also enhanced learning ability ($P < 0.03$) and m mory retention ($P < 0.01$) in brain-injured animals at 2 weeks postinjury. Taken together, these data suggest that chronic, posttraumatic administration of the trophic factor IGF-1 may be efficacious in ameliorating neurobehavioral dysfunction associated with traumatic brain injury. © 1997 Academic Press

INTRODUCTION

Insulin-like growth factor-1 (IGF-1) is known to mediate many of the cellular and molecular effects formerly attributed solely to growth hormone (62). While its systemic actions are primarily metabolic, IGF-1 also influences cellular differentiation and mitogenic activity (25, 70). In the central nervous system (CNS), endogenous IGF-1 appears to function as a trophic factor whose biological actions are mediated by the membrane-bound IGF-1 receptor (3). IGF-1 gene expression in the brain is developmentally regulated and has been localized to both neurons and glia (1, 5). During a time of neuronal differentiation and neurite outgrowth, IGF-1 and IGF-1 receptors are expressed in high concentrations, after which the number of IGF receptors decreases with increasing age and maturation of the CNS (1, 4, 5, 41). The expression of IGF-1 receptors in regions where IGF-1 is synthesized is suggestive of a local autocrine or paracrine mode of action of IGF-1 (6, 40).

The trophic and reparative properties of IGF-1 have been demonstrated in numerous *in vitro* studies. In cell cultures, IGF-1 enhances neuronal survival and differentiation (7, 36, 48, 68) and has potent mitogenic effects on both neurons and glia (26, 39, 47). Neurite outgrowth in neuronal cultures is promoted by exogenous IGF-1 (9, 58, 74) and appears to be coincident with IGF-1-induced elevations in mRNA levels for selected cytoskeletal proteins (19, 73). IGF-1 has also been reported to protect cultured neurons against both iron-induced neurotoxicity and calcium-mediated hypoglycemic and hypoxic damage by preventing the loss of intracellular calcium homeostasis and mitochondrial transmembrane potential (11, 43, 77).

In conjunction with these *in vitro* studies, IGF-1 has been evaluated as a potential neuroprotective agent in several *in vivo* models. Following peripheral nerve injury, local infusion of IGF-1 has been shown to stimulate axonal regeneration, enhance functional recovery, and increase nerve sprouting (9, 14, 27, 33, 65), while antibodies to native IGF-1 have been reported to inhibit axonal regeneration (33). Intracerebroventricular injection of IGF-1 markedly reduced neuronal loss in injured areas following experimental hypoxic ischemia (22–24) and transient forebrain ischemia (78). The effects of IGF-1 were determined to be mediated by the IGF-1 receptor, independent of systemic glucose concentrations or brain temperature (23). In the present study, we have evaluated the potential efficacy of systemic IGF-1 administration in the treatment of both cognitive and neurological motor dysfunction following lateral fluid percussion (FP) brain injury in the rat. To our knowledge, this is the first study of IGF-1 treatment after traumatic brain injury as well as the first demonstration of cognitive enhancement by exogenous IGF-1.

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MATERIALS AND METHODS

Surgical Procedures and Drug Infusion

In Study 1, male Sprague–Dawley rats (n = 30) weighing 345–385 g were subjected to lateral FP brain injury as previously described (46). Briefly, animals were anesthetized with sodium pentobarbital (60 mg/kg, ip) and a 5-mm craniectomy was performed over the left parietal cortex. The animal was attached to the FP injury device via a hollow Luer-lok fitting cemented into the craniectomy site. Lateral FP brain injury was induced by the rapid injection of saline through the sealed fitting. All animals received a brain injury of moderate severity (2.4–2.6 atm). After injury, the fitting was removed and the skin was sutured. Normothermia was maintained with warming pads until the animals began to ambulate. Brain temperature was not directly monitored because the use of warming pads maintains normal brain temperature in this model of brain trauma (52). Fifteen minutes following brain injury, surviving animals randomly received a subcutaneous injection of either recombinant human IGF-1 (Cephalon, West Chester, PA) (1.0 mg/kg, n = 10) or vehicle (100 mM acetic acid, pH = 4.0; n = 15). Injections were then given twice daily at 12-h intervals over a 14-day period. The dose of 1 mg/kg was chosen based on consultation with Cephalon scientists and on a previous dose–response study in mice (14).

In Study 2, male Sprague–Dawley rats (n = 58) weighing 370–425 g were anesthetized and either subjected to moderate severity FP brain injury (2.5–2.9 atm; n = 42) or surgically prepared without brain injury to serve as uninjured controls ("shams," n = 16). Fifteen minutes following brain injury, osmotic minipumps (Model 2ML2, Alza, Palo Alto, CA) containing either recombinant human IGF-1 (10 mg/ml, pH = 4.0; n = 15 injured, n = 8 sham) or vehicle (100 mM acetic acid, pH = 4.0; n = 13 injured, n = 8 sham) were implanted subcutaneously into surviving animals which were assigned to each group in a blinded fashion. The minipumps were primed by immersion in 37°C saline for 4 h prior to implantation. Thus, animals received a continuous infusion of either vehicle or approximately 4 mg/kg/day of IGF-1 over 2 weeks. Initial trials for Study 2 were performed using continuous infusion of 8 mg/kg/day which, in control rats, results in steady state plasma IGF-1 levels comparable to maximum levels achieved with subcutaneous injections of 1 mg/kg (716 ± 34 ng human IGF-1/ml). However, since the 8 mg/kg/day dosing was not well tolerated by brain-injured rats (see Results), a lower dose of 4 mg/kg/day was selected. Previous studies have shown that plasma concentrations of human IGF-1 vary linearly with dosing (14).

Neurobehavioral Motor and Cognitive Paradigms

In Study 1, neurological motor function for each animal was evaluated at the same time each day for the 14-day drug administration period following brain injury. A baseline level of function was evaluated on the day prior to injury. Evaluation of posttraumatic neurological motor function was performed by an experienced investigator, blinded to treatment, using a previously described composite of tests (46). These motor tests have been shown to correlate with injury severity and are known to be sensitive to pharmacological manipulation (46, 52). Animals were given an integer score from 0 (severely impaired) to 4 (normal) for each of the following six indices: left and right forelimb flexion during suspension by the tail, left and right hindlimb flexion when the forelimbs remain on a hard surface and the hindlimbs are lifted up and back by the tail, and the ability to resist lateral pulsion toward the left and right. In addition, animals were tested for their ability to stand on an inclined plane (angle board) while facing left, right, and upward. Scores (0–4) were assigned for each direction according to their difference from preinjury performance and then averaged for inclusion in the composite score. A composite neuromotor score (0–28) was generated by combining the scores for each of these seven tests.

Study 2 was designed to corroborate the neurological motor function data from Study 1, using a more convenient, continuous drug administration paradigm. To this end, neurological motor function was evaluated at 2, 7, and 14 days following brain injury using the composite score described above. Additional motor function data were obtained by testing animals using a beam walking task, performed as previously described (61), to evaluate complex motor movement and coordination. Animals were acclimated to the task on each of 2 days prior to injury. Following injury, their performance in the third of three trials on each day was scored from 0 (afunctional) to 7 (normal).

In light of the observed improvement in motor function following IGF-1 treatment in Study 1, the behavioral analysis was purposefully expanded in Study 2 to include evaluation of cognitive function. Animals were tested in the Morris water maze (MWM), a 1-m-diameter tank filled with 18°C water, using an acquisition, or visuospatial learning, paradigm (56). Over a series of 10 trials on each of Days 13 and 14 after injury, animals learned the location of a submerged platform using visual cues outside the maze. Acclimation to the task occurs predominantly on the first day of training. The time (latency) to reach the hidden platform was recorded for each trial. Improvements in posttraumatic learning latencies have been demonstrated following pharmacological manipulation using this paradigm (56). On Day 15 following brain injury, the platform was removed from the MWM and animals were allowed to

swim for 60 s to evaluate their memory of the platform location (probe trial). Their swimming patterns were recorded by video and analyzed to assign a score based on the time spent in specific regions of the maze, giving more weight to those regions closest to the prior platform location (66). In addition, the swimming distance and average swim speed were calculated for each animal during the probe trials.

Physiological Measurements

All animals were housed in a vivarium on a 12-h light/dark cycle and allowed access to both food and water *ad libitum* before and after surgical preparation and brain injury. In Study 1, each animal's body weight was recorded on the day of surgery, and at 7 and 14 days following injury. In Study 2, body weight was recorded for each animal at five intervals: 2 days prior to surgery, on the day of surgery, and at 2, 7, and 14 days postinjury. Blood glucose concentrations were measured in all animals in Study 2 at 90 min and at 1, 2, 8, and 15 days following brain injury. Blood samples were obtained from the rat's tail, dropped onto a glucose test strip, and analyzed using a blood glucose meter (Ames Glucometer Encore, Miles Inc., Elkhart, IN).

Statistical Analysis

Neurological motor scores are considered to be ordinal data and are not normally distributed. Therefore, these data were evaluated using individual Mann–Whitney *U* tests. However, to allow multiple comparisons of the neurological motor scores taken daily in Study 1, these particular data were also analyzed using a repeated measures one-way ANOVA. Learning latencies, probe trial scores, and swimming distances and speeds are considered parametric data and were analyzed using a two-way ANOVA followed by a Bonferroni *t* test. Learning latencies were analyzed for each day of training and for all 20 trials combined, as reported previously (56). Mean values for blood glucose concentrations and body weights were analyzed using a repeated measures two-way ANOVA followed by pairwise comparisons with a Bonferroni correction. For analysis of behavioral outcome measures, animals whose scores fell outside two standard deviations from the mean of the group were excluded. A P value of less than 0.05 was considered statistically significant. Mean values are reported with standard errors (SEM).

RESULTS

Study 1: Subcutaneous Administration at 12-h Intervals

Of the 30 animals which received brain injury, 25 survived to receive treatment at 15 min postinjury. This mortality rate is typical for lateral FP brain injury of moderate severity (46). Subsequent to initiation of treatment, one animal receiving vehicle died (approximately 3 h postinjury) and one animal receiving IGF-1 died (2 days postinjury), leaving 14 vehicle treated animals and 9 IGF-1 treated animals for behavioral analysis.

At 1 and 2 days postinjury, the neurological motor deficit demonstrated by IGF-1 treated animals was comparable to that shown by the vehicle treated animals (Fig. 1). However, animals receiving IGF-1 showed significantly improved motor function evaluated over the 2-week period ($P < 0.02$, one-way ANOVA). When compared to vehicle treatment on individual days, IGF-1 treatment resulted in a significant attenuation of motor dysfunction at 5, 7, 12, and 14 days postinjury ($P < 0.05$) and a similar trend at 6 and 10 days following brain injury ($P < 0.10$) (Fig. 1). Significant differences between IGF-1 and vehicle treated animals were also observed for the seven individual motor tests, in particular for the hindlimb flexion and right forelimb flexion tests (data not shown).

Prior to injury no significant differences in body weight were observed between animals that later received vehicle (364 ± 3 g, mean ± SEM) and IGF-1 (371 ± 3 g). At 7 days following brain injury, vehicle treated animals weighed significantly less (332 ± 6 g) than IGF-1 treated animals (360 ± 9 g, $P < 0.02$). This trend continued out to 14 days, at which time IGF-1 treated, brain-injured animals weighed 387 ± 13 g and vehicle treated animals weighed 356 ± 8 g ($P < 0.05$).

Study 2: Continuous Subcutaneous Administration

Of the 42 animals which received brain injuries in Study 2, 28 survived until implantation of Alzet pumps

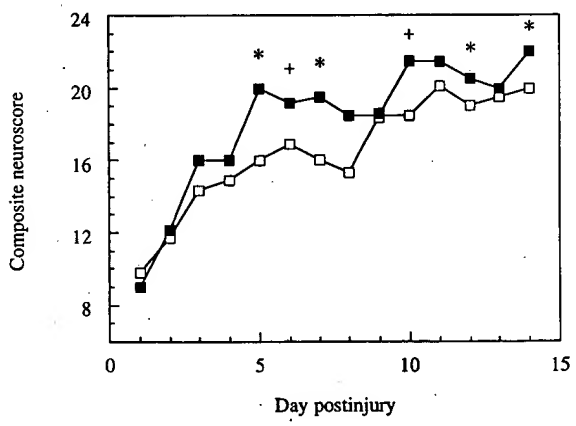

FIG. 1. Composite neurological motor function measured daily for 2 weeks following lateral fluid percussion brain injury. In Study 1, animals were treated every 12 h with either 1 mg/kg IGF-1 (filled squares) or an equal volume of vehicle solution (open squares). Symbols represent median scores on a scale of 0–28. *$P < 0.05$ and +$P < 0.10$, when compared to vehicle treated animals on the same day.

at 15 min postinjury. One brain-injured animal which was receiving IGF-1 died within the first 24 h postinjury. In addition, one IGF-1 treated, brain-injured animal exhibited severe prolonged hypoglycemia, was euthanized at 8 days postinjury, and was excluded from all analyses. Therefore, 13 vehicle treated and 13 IGF-1 treated, brain-injured animals remained for behavioral analysis.

Lateral FP brain injury caused profound motor dysfunction in vehicle treated animals relative to uninjured controls at 2 days after injury ($P < 0.001$; Fig. 2A). While some spontaneous recovery is typically observed in vehicle treated, brain-injured animals over the first 2 weeks postinjury, significant neurological motor deficits persisted at both 7 days ($P < 0.001$; Fig. 2B) and 14 days ($P < 0.001$; Fig. 2C). In fact, at each time point, brain injury resulted in a highly significant deficit independent of treatment (main effect of injury, $P < 0.001$). In brain-injured animals, no significant treatment effect was observed at 2 or 7 days. However, following 14 days of continuous IGF-1 administration, a significant improvement in motor function was observed in brain-injured animals ($\chi^2 = 4.18$, $P < 0.05$; Fig. 2C). Analysis of the individual neurological motor function scores revealed that the right hindlimb flexion and right forelimb flexion tests were most sensitive in detecting the beneficial effects of chronic IGF-1 administration (Fig. 3). The beam walking ability of vehicle treated, brain-injured animals was significantly impaired at 2, 7, and 14 days postinjury (median scores of 3, 5, and 5, respectively) when compared to uninjured animals (median scores of 7, 7, and 7) ($P < 0.001$ at each time). Continuous administration of IGF-1 did not improve animals' beam walking ability after trauma (median scores of 3, 5, and 6).

Fluid percussion brain injury produced a significant learning deficit which was evident on Days 13 and 14 postinjury. Uninjured animals showed a rapid reduction in latency over their first 10 trials in the MWM and retained their ability to find the platform site on the next day, consistently exhibiting latencies of 5 s or less by the end of the 20 trials (Fig. 4A). In contrast, during 20 trials brain-injured animals showed a sustained impairment in learning the platform location (Fig. 4B). Two-way analysis of variance revealed a significant main effect of brain injury for trials 1–10 and significant main injury as well as treatment effects for trials 11–20 and 1–20. Brain-injured animals receiving vehicle had significantly longer latencies than did sham animals during trials 1–10 ($P < 0.005$), trials 11–20 ($P < 0.005$), and trials 1–20 ($P < 0.001$) (Fig. 5). Administration of IGF-1 resulted in a marked improvement in posttraumatic learning ability in trials 1–20 ($P < 0.03$ compared with vehicle treated, brain-injured animals), most notably in trials 11–20 ($P < 0.02$). IGF-1 treatment did not significantly affect the learning ability of uninjured (sham) animals.

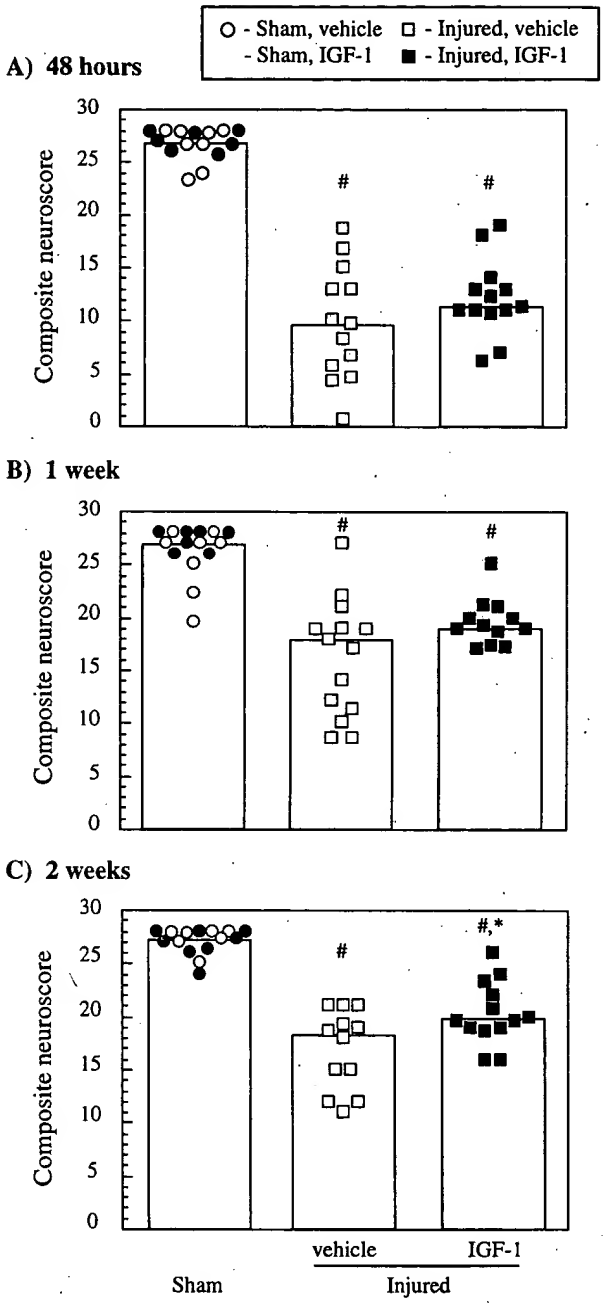

FIG. 2. Composite motor function scores for animals receiving continuously administered 4 mg/kg/day IGF-1 or vehicle when tested at (A) 48 h, (B) 1 week, and (C) 2 weeks following FP brain injury. Individual animals' scores are represented by circles (uninjured) or squares (injured). Filled symbols indicate IGF-1 treatment and open symbols indicate vehicle treatment. Bars represent median scores. #$P < 0.001$ when compared to sham animals; *$P < 0.05$ when compared to vehicle treated injured animals.

Data from a probe trial performed on Day 15, analyzed with two-way ANOVA, showed that there was a significant effect of brain injury ($P < 0.005$) as well as a

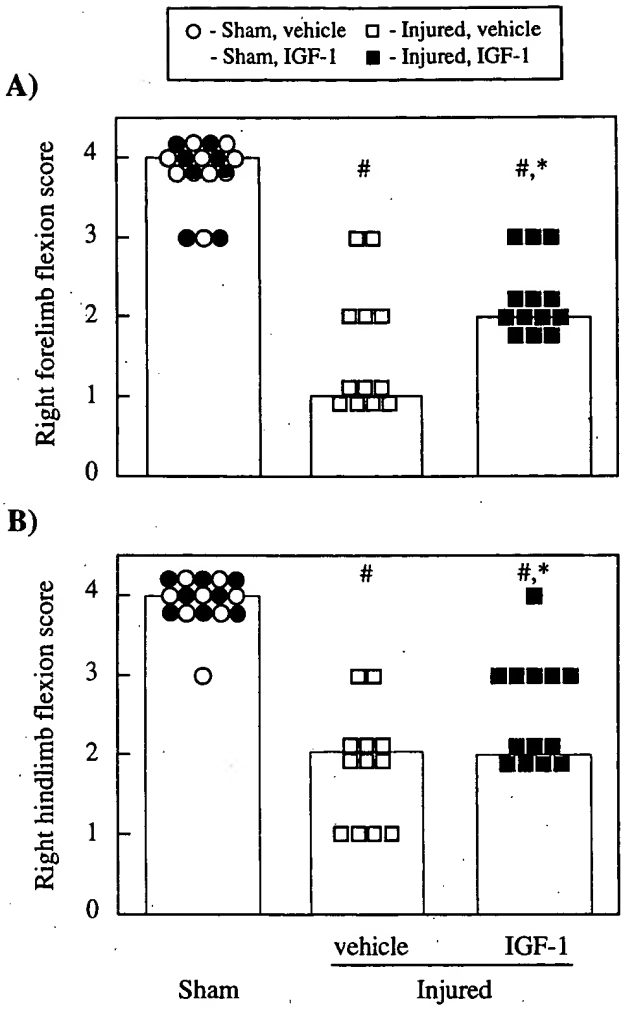

FIG. 3. Individual motor function test scores at 2 weeks postinjury for animals receiving either continuously administered IGF-1 or vehicle: (A) Right forelimb flexion test, (B) right hindlimb flexion test. Individual animals' scores are represented by circles (uninjured) or squares (injured). Filled symbols indicate IGF-1 treatment and open symbols indicate vehicle treatment. Bars represent median scores. #$P < 0.001$ when compared to sham animals; *$P < 0.05$ when compared to vehicle treated injured animals.

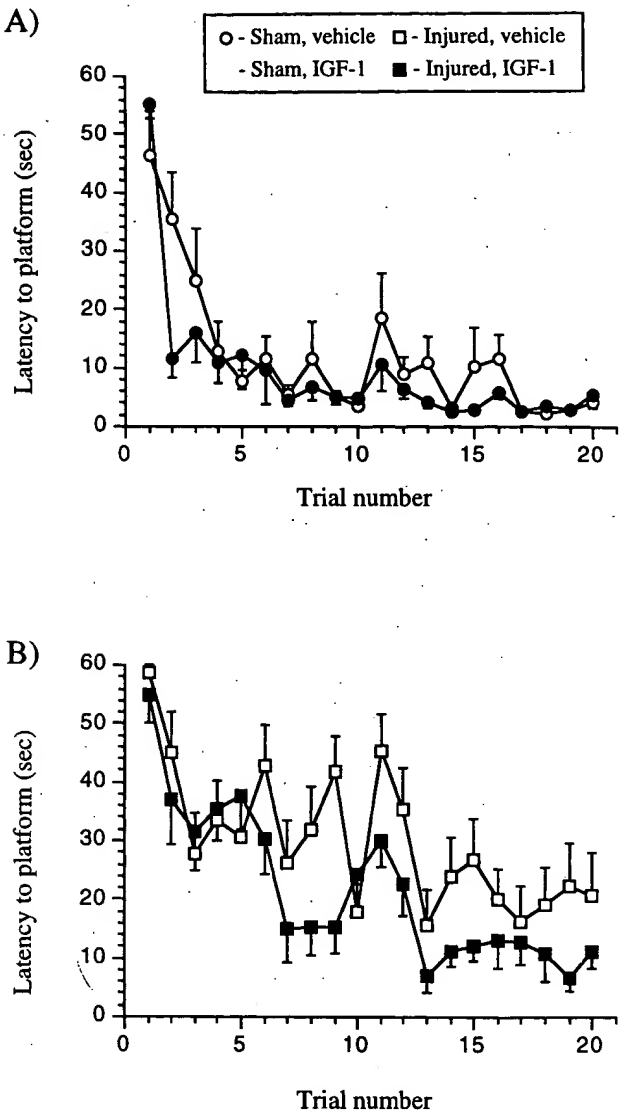

FIG. 4. Learning curves for animals trained in the MWM on Days 13 and 14. (A) Both vehicle treated (open circles) and IGF-1 treated (filled circles) uninjured animals learned the platform location quickly, exhibiting a rapid reduction in latency (s) over 20 training trials. (B) Injured animals exhibited longer latencies than uninjured animals, indicating impaired ability to learn the platform location. IGF-1 administration (filled squares) attenuated the impairment observed in brain-injured animals compared to vehicle administration (open squares). Symbols represent mean latencies (maximum, 60 s); bars represent standard errors.

significant interaction between injury status and treatment ($P < 0.01$) (Fig. 6). Vehicle treated, brain-injured animals challenged to recall the platform location performed substantially worse than uninjured animals treated with either vehicle ($P < 0.001$), or IGF-1 ($P < 0.05$). IGF-1 treated brain-injured animals showed improved memory retention when compared to vehicle treated injured animals ($P < 0.01$, t test) and were not significantly different from either sham group. As in the learning trials, IGF-1 treatment did not significantly alter the performance of sham animals. The average swimming speeds during the probe trials were not significantly affected by either injury or IGF-1 treatment, indicating that brain-injured animals perform normally on a basic motor task such as swimming, despite deficits in more complex tasks such as beam walking. Uninjured animals receiving either vehicle or IGF-1 swam at average speeds of 40.1 ± 1.6 and 41.9 ± 2.2 cm/s (mean ± SEM), respectively. Vehicle and IGF-1 treated brain-injured animals swam at average speeds of 36.6 ± 1.3 and 37.2 ± 1.2 cm/s, respectively.

Although all vehicle treated animals lost weight in the 2 days following anesthesia and surgery, uninjured animals tended to return to their baseline weight within 1–2 weeks (Fig. 7). Brain injury exacerbated

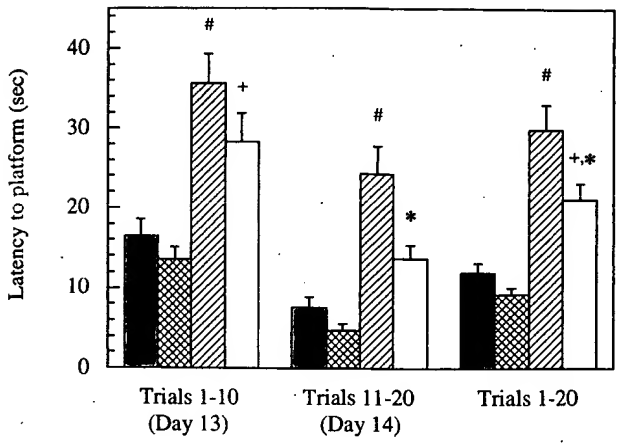

FIG. 5. Effect of chronic IGF-1 administration on learning latencies. Mean latencies (in s) are shown for the block of 10 trials given on Day 13, the block of 10 trials given on Day 14, and the complete set of 20 trials. Filled bars show latencies for uninjured animals receiving vehicle; cross-hatched bars show uninjured, IGF-1 treated animals' latencies. Single hatched bars represent latencies for vehicle treated, injured animals and open bars show IGF-1 treated, injured animals' latencies. Error bars show SEM. #$P < 0.005$ when compared to vehicle treated, uninjured animals; +$P < 0.05$ when compared to IGF-1 treated, uninjured animals; *$P < 0.05$ when compared to vehicle treated, injured animals.

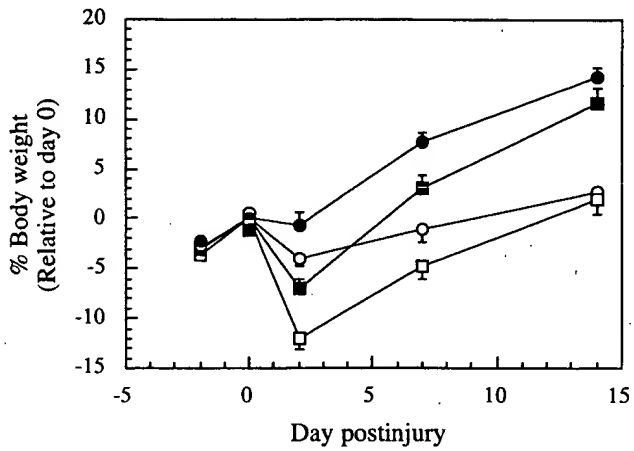

FIG. 7. Effect of brain injury and IGF-1 treatment on body weight. Over a 2-week period following injury, sham animals (circles) weighed significantly more than injured animals (squares) receiving equivalent treatment ($P < 0.005$). Administration of 4 mg/kg/day of IGF-1 (filled symbols) was associated with greater weight gain over the 2-week period than administration of vehicle (open symbols) ($P < 0.001$). Weights were calculated as a percentage weight gain relative to the animal's weight on the day of surgery. Means are plotted with SEM.

postsurgery weight loss for both vehicle and IGF-1 treated animals ($P < 0.005$). However, vehicle treated, brain-injured animals returned to their preinjury weight within approximately 14 days. Chronic administration of IGF-1 promoted significant weight gain in both injured and sham animals over time ($P < 0.001$) and when compared to administration of vehicle ($P < 0.001$).

In animals receiving vehicle, blood glucose concentrations were not influenced by FP brain injury (Table 1). Treatment with IGF-1 resulted in a slight but nonsignificant decline in blood glucose levels in sham animals. However, superposition of FP brain injury with IGF-1 administration resulted in mild, but significant, hypoglycemia at 1 day after injury ($P < 0.05$ compared to IGF-1 treated shams, $P < 0.001$ compared to vehicle treated shams). Subsequent to 1 day postinjury animals were able to maintain blood glucose levels within a normal range despite chronic infusion of IGF-1, suggesting that hypoglycemia was a transient phenomenon following the initiation of IGF-1 administration. All groups exhibited an elevation in blood glucose concentration at 90 min following FP brain injury or

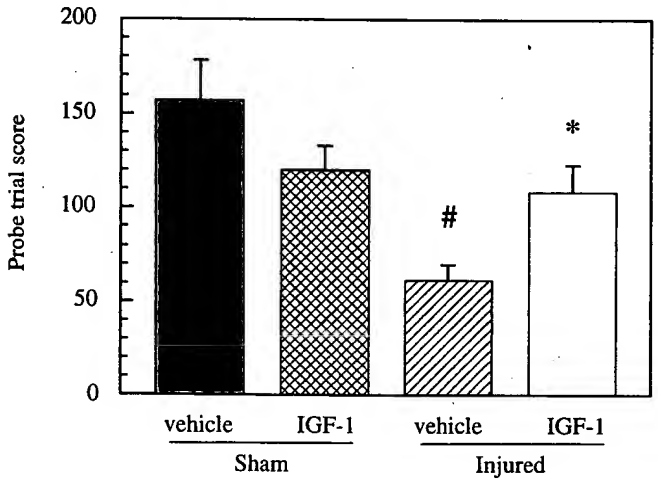

FIG. 6. Probe trial scores received on Day 15 assess the ability to recall the platform location learned on Days 13 and 14 in the MWM. On Day 15, the platform was removed and animals were scored during a 60-s swimming trial according to the amount of time spent swimming in proximity to the previously learned platform location. IGF-1 was administered at 4 mg/kg/day for 14 days postinjury. Bars represent mean scores with SEM. #$P < 0.05$ when compared to either group of sham animals; *$P < 0.01$ when compared to vehicle-treated injured animals.

TABLE 1

Effect of Brain Injury and IGF-1 Treatment on Blood Glucose

	Blood glucose concentration (mg/dl)				
Group	90 min	1 Day	2 Day	8 Day	15 Day
Sham, vehicle	126 ± 5	110 ± 5	107 ± 5	112 ± 3	104 ± 2
Sham, IGF-1	132 ± 6	98 ± 5	100 ± 7	99 ± 2	106 ± 3
Injured, vehicle	134 ± 6	110 ± 4	107 ± 4	106 ± 2	106 ± 1
Injured, IGF-1	140 ± 6	76 ± 5*	92 ± 6	111 ± 8	111 ± 2

Note. Animals were given vehicle or 4 mg/kg/day of IGF-1 for 14 days postinjury. Numbers are means ± SEM.

*$P < 0.001$ when compared to either vehicle treated group at 1 day and $P < 0.05$ when compared to IGF-1 treated sham group at 1 day.

sham injury (control animals). In preliminary investigations, higher doses of IGF-1 (5.0 mg/kg in the twice daily injection protocol and 8 mg/kg/day in the continuous infusion protocol) resulted in severe prolonged hypoglycemia (typically, less than 40 mg/dl over 1–2 days postinjury) in brain-injured animals, which was associated with one or more of the following: spasms, impaired hindlimb mobility, excessive weight loss, and mortality. Uninjured animals tolerated these higher doses of IGF-1 without any noticeable side effects.

DISCUSSION

Animals subjected to lateral FP brain injury and treated with vehicle for 14 days postinjury demonstrated significant motor and cognitive (learning) deficits. Previous work in our laboratory has shown that these behavioral deficits persist for months following FP brain injury in rats (46, 57, 66). Trauma-induced neurological motor deficits were significantly attenuated by both periodic and continuous systemic administration of IGF-1 for 14 days following brain injury. Furthermore, treatment with IGF-1 significantly enhanced the ability of brain-injured animals to learn a visuospatial task after trauma.

Although large proteins do not normally cross the intact blood–brain barrier (BBB), studies have demonstrated that IGF-1 (molecular weight approximately 7000 (4)) crosses the BBB in rats (59). The transcytosis of IGF-1 through the BBB is mediated by IGF-1 receptors localized in brain capillaries (55). In the present studies, the availability of systemically administered IGF-1 to injured brain regions may have been facilitated by the prolonged opening (several days to 1 week) of the BBB which occurs following traumatic brain injury in rats (2, 17, 32, 69). The administration of IGF-1 for 2 weeks postinjury encompassed the period of BBB disruption, thereby maximizing the availability of this trophic factor to the injured brain.

Weight loss following FP brain injury may result from reduced food and water intake following surgery as well as from catabolic processes initiated by the brain injury. Our observations that subcutaneous administration of IGF-1 over 2 weeks was associated with increased body weight were consistent with data demonstrating that IGF-1 attenuates diet-induced weight loss in rats (70) and promotes weight gain following surgery (38). In the present study, IGF-1 administration also induced transient, mild hypoglycemia in brain-injured, but not uninjured, animals. Following moderate FP brain injury in rats, local cerebral glucose metabolism rates have been reported to be transiently elevated for up to 6 h, after which time prolonged hypometabolism occurs (16, 75). Administration of IGF-1 during a postinjury hypermetabolic state may contribute to the mild hypoglycemia observed in brain-injured but not sham animals. Head-injured patients are known to suffer from hypermetabolism, hypercatabolism, and hyperglycemia (12, 76), suggesting that the ability of IGF-1 treatment to reduce blood glucose levels and increase body weight following experimental brain injury may be beneficial.

Despite the mild systemic effects observed with IGF-1 administration, we believe that the neurological motor and cognitive improvements observed following brain injury in this study are largely due to direct effects of IGF-1 on the CNS. The battery of neurological motor function tests is composed primarily of reflexive tasks, scored to a large degree on quality, extent, and speed of movement and to a lesser degree on strength of movement. Within each of the four experimental groups there was no clear trend in the behavioral outcome measures as a function of body weight. Furthermore, IGF-1 treated animals swam at speeds equivalent to those measured for vehicle treated animals in the MWM, suggesting that the observed neurobehavioral effects were independent of the body weight of the animal. Similarly, the behavioral efficacy of IGF-1 administration is unlikely to be a result of hypoglycemia. A recent study comparing normo-, hyper-, and hypoglycemic rats subjected to FP brain injury revealed no significant differences in neurological motor function at 1 and 2 weeks postinjury (71).

We have demonstrated that chronic treatment with the trophic factor IGF-1 can significantly improve posttraumatic motor function in rats. In rats assessed daily for neurological motor function, IGF-1 treatment over 2 weeks attenuated motor dysfunction, although the effect was somewhat delayed, becoming significant only after 5 or more days of treatment. Chronic, subcutaneous IGF-1 treatment has been observed to enhance the rate of recovery of grip strength and gait in mice subjected to sciatic nerve crush (14). In addition, IGF-1 administered daily over a 6-week period to mutant mice exhibiting motoneuron degeneration led to increased grip strength only after 3 or more weeks of treatment (28). Together, these data suggest that chronic treatment with this growth factor may be important for long term improvements in motor function.

In addition to attenuating posttraumatic neurological motor deficits, IGF-1 treatment significantly improved the visuo-spatial learning ability of brain-injured rats at 2 weeks following trauma. Furthermore, at 1 day following the learning trials, the ability of brain-injured animals receiving IGF-1 to recall the learned task was superior to that of animals receiving vehicle. To our knowledge, the effects of IGF-1 on cognition in models of CNS injury have not been previously investigated. However, insulin has been shown to ameliorate cognitive deficits observed in models of cerebral ischemia, when administered both prior to (67) and following (72) an ischemic insult. In

addition, improvements in posttraumatic cognitive function have been demonstrated using other neurotrophic factors. When infused directly into the injured cortex beginning at 24 h postinjury, both nerve growth factor (NGF) (64) and basic fibroblast growth factor (bFGF) (44) attenuate memory deficits evaluated at 1 week following lateral FP brain injury in rats. These data demonstrate that trophic factors have therapeutic potential even when treatment is substantially delayed and suggest that a long window of opportunity may also be available for the initiation of treatment with IGF-1.

The beneficial effects of IGF-1 on posttraumatic neurobehavioral deficits may be related to several neuroprotective effects of this trophic factor that have been documented in the CNS. The pathogenesis of traumatic brain injury has been linked, in part, to the marked release of excitatory amino acid (EAA) neurotransmitters and a pathologic increase in intracellular calcium which can stimulate the genesis of free radicals and activate proteolytic enzymes (45). Studies have documented trauma-induced perturbations in calcium (20, 49, 51, 63) as well as an acute and significant increase in extracellular glutamate concentrations following both experimental (18, 34, 50, 53) and clinical (8) brain trauma. Accumulation of iron, which is known to damage cells by catalyzing the peroxidation of proteins and lipids, has been reported to occur following experimental brain trauma (54). *In vitro,* IGF-1 protects neurons from *N*-methyl-D-aspartate (NMDA)-induced toxicity (48), glucose deprivation (11, 43), and hypoxia (43) and increases the levels of the calcium binding proteins calretinin and calbindin D-28k (74). IGF-1 has also been shown to protect cultured neurons against iron-induced damage (77).

Under normal conditions, glial cells in the CNS provide trophic support for neurons. A marked proliferation of both astrocytes (reactive astrocytosis) and microglia has been shown to occur within the first week following experimental brain trauma (29, 31, 35). IGF-1 is known to stimulate glial cell proliferation (26, 39). Although chronic IGF-1 administration may influence glia in the posttraumatic environment, it is not clear whether increased glial reactivity is beneficial or harmful to the injured brain. However, recent studies suggest a role for IGF-1 in the regulation of myelination (10, 37), which might be beneficial following brain injury.

Behavioral deficits resulting from lateral FP brain injury are accompanied by regional necrotic as well as apoptotic cell death which progresses over many weeks postinjury (13, 15, 30, 60). IGF-1 is known to promote the *in vitro* survival of many different populations of CNS neurons (7, 36, 48, 68) and has been shown to inhibit apoptotic cell death in cultured cerebellar granule cells (21) and neuroblastoma cells (42). Administration of exogenous IGF-1 directly to the CNS has been reported to rescue damaged neurons and prevent death of compromised neurons subjected to hypoxic–ischemic injury or transient forebrain ischemia in rats (22–24, 78). In addition to promoting neuronal survival, IGF-1 enhances neurite outgrowth *in vitro* (9, 74), while concomitantly increasing levels of neurofilament and tubulin mRNA (19, 73). In light of the trophic properties of IGF-1, it is possible that behavioral improvements observed due to posttraumatic administration of IGF-1 in the present study may be accompanied by increased cell survival.

In conclusion, we have demonstrated that prolonged, systemic administration of IGF-1 after lateral FP brain injury attenuates both posttraumatic neurological motor and cognitive deficits in rats. This trophic factor may provide a novel means for treating behavioral dysfunction following traumatic brain injury.

ACKNOWLEDGMENTS

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Selective and Nonselective Stimulation of Central Cholinergic and Dopaminergic Development *in vitro* by Nerve Growth Factor, Basic Fibroblast Growth Factor, Epidermal Growth Factor, Insulin and the Insulin-like Growth Factors I and II

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To study the selectivity of neurotrophic actions in the brain, we analyzed the actions of several known growth factors on septal cholinergic, pontine cholinergic, and mesencephalic dopaminergic neurons in culture. Similar to nerve growth factor (NGF), basic fibroblast growth factor (bFGF) stimulated choline acetyltransferase activity in septal cultures. In contrast to NGF, bFGF also enhanced dopamine uptake in mesencephalic cultures and stimulated cell proliferation in all 3 culture types. Insulin and the insulin-like growth factors I and II stimulated transmitter-specific differentiation and cell proliferation in all culture types. Epidermal growth factor (EGF) produced a small increase in dopamine uptake by mesencephalic cells and stimulated cell proliferation in all culture types. In septal cultures, bFGF was most effective when given at early culture times, NGF at later times. The stimulatory actions of bFGF and insulin did not require the presence of glial cells and were not mediated by NGF. In mesencephalic cultures, the stimulation of dopamine uptake by bFGF and EGF was dependent on glial proliferation. The results suggest different degrees of selectivity of the neurotrophic molecules. NGF and, very similarly, bFGF seem to influence septal cholinergic neurons directly and rather selectively, whereas the neurotrophic actions of insulin and the insulin-like growth factors appear to be more general.

It is widely believed that for a given neuron or neural connection to survive during development, proper contact with the projection area has to be established. The known biology of nerve growth factor (NGF) supports the concept that such neuron-target interactions are based on the production and release of specific trophic molecules by the target area which are required by the innervating neurons (for review, see Thoenen and Edgar, 1985; Purves, 1986; Thoenen et al., 1987; Barde, 1989). Besides NGF, several other substances or factors have been characterized that are able to support survival or differentiation of neurons. Ciliary neurotrophic factors (CNTF) have been isolated from chick eye (Barbin et al., 1984), rat sciatic nerve (Manthorpe et al., 1986), and bovine heart (Watters and Hendry, 1987) based on their ability to provide survival of chick embryonic ciliary ganglion cells in culture. A brain-derived neurotrophic factor (BDNF) has been purified from pig brain (Barde et al., 1982) and promotes survival and fiber outgrowth of embryonic chick sensory neurons and rat retinal cells (Davies et al., 1986; Johnson et al., 1986; Barde et al., 1987; Kalcheim et al., 1987; Hofer and Barde, 1988). Furthermore, substances that were previously known for their effects on non-neuronal tissues have recently been recognized to display neurotrophic effects when tested in cell culture assays. Among these molecules are basic fibroblast growth factor (bFGF), insulin, the insulin-like growth factors I and II (IGF-I, IGF-II), and epidermal growth factor (EGF). bFGF has been shown to promote survival and fiber outgrowth of dissociated mouse and rat fetal neurons (Morrison et al., 1986; Walicke et al., 1986; Unsicker et al., 1987; Walicke, 1988) and to stimulate neurite formation in PC12 cells (Togari et al., 1985; Rydel and Greene, 1987). According to a recent report, bFGF also stimulates the proliferation of neuronal precursor cells in culture (Gensburger et al., 1987). In support of a possible function of bFGF in the CNS, bFGF activity and immunoreactivity and receptor for bFGF have been found in the brain (Logan and Logan, 1986; Imamura et al., 1988; Presta et al., 1988).

A neuronal bFGF receptor with properties distinct from the mesenchymal receptor has recently been characterized (Walicke et al., 1989). Suggestive of many different roles of this molecule in the mammalian organism is the fact that as many as 10 previously characterized growth factors are probably similar or identical to bFGF (Gospodarowicz et al., 1986). Insulin, IGF-I, and IGF-II have only recently been suggested to possess physiological functions for the CNS (for review, see Baskin et al., 1988). Receptors for insulin and the IGFs are found in the rat brain and seem to be heterogeneously distributed (Hill et al., 1986; Mendelsohn, 1987; Bohannon et al., 1988). Evidence that insulin occurs in the brain is still equivocal (Baskin et al., 1988), but mRNAs for IGF-I and IGF-II were detected in many brain areas and seem to be differentially regulated during development (Rotwein et al., 1988). In cultures of brain cells insulin, IGF-I, and IGF-II promote neuronal survival, neurite extension, expression of neuronal enzymes, and, in astrocytes but possibly also in neurons, DNA synthesis (Bhat, 1983; Lenoir and Ho-

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negger, 1983; Mill et al., 1985; Recio-Pinto et al., 1986; Aizenman and DeVellis, 1987; Kyriakis et al., 1987; Avola et al., 1988; DiCicco-Bloom and Black, 1988). Mild increases in dopamine uptake of ventral mesencephalic cultures by IGF-I and IGF-II have been reported recently (Valdes et al., 1988). EGF is primarily known as a potent mitogen for several cell types including astrocytes (Schlesinger et al., 1983; Avola et al., 1988) but, similar to bFGF, has recently been shown to promote neuronal survival and neurite outgrowth of dissociated neonatal rat brain cells in serum-free medium (Morrison et al., 1987, 1988). The EGF molecule is structurally and functionally related to transforming growth factor alpha (Anzano et al., 1982; Marquardt et al., 1984), which is synthesized in the brain (Wilcox and Derynck, 1988). Both molecules act on the same receptors (Massague, 1983). Some of the non-neuronal actions of EGF are shared by platelet-derived growth factor (PDGF; Cooper et al., 1982) and PDGF has recently been shown to play a role in glial differentiation (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988).

The concept of NGF as an instrument of target-controlled neuronal survival has been established in the PNS. The exact role of NGF in the CNS remains to be fully elucidated. Presently, actions of NGF on the cholinergic neurons of the basal forebrain and the corpus striatum are well characterized (for reviews, see Thoenen et al., 1987; Whittemore and Seiger, 1987; Hefti et al., 1989), and distribution and developmental changes of NGF and its receptor in the CNS have been extensively investigated (for recent studies, see Ayer-LeLievre et al., 1988; Buck et al., 1988; Ernfors et al., 1988; Hefti and Mash, 1988; Kiss et al., 1988; Schatteman et al., 1988; Yan and Johnson, 1988). All data are compatible with the view that NGF serves as a target-derived survival factor for basal forebrain neurons. However, it is still not known whether there is neural cell death during the development of the basal forebrain or striatal cholinergic system and whether the availability of NGF regulates the number of cholinergic neurons. Furthermore, the selectivity of the action of NGF in the CNS remains one of the principal unresolved questions. While the distribution of NGF and its receptor suggests actions of NGF on neurons other than cholinergic (Buck et al., 1988; Ernfors et al., 1988; Schatteman et al., 1988; Yan and Johnson, 1988; Large et al., 1989), no other NGF-responsive central populations have yet been identified with certainty. Far less is known about the other neurotrophic factors and their functions in the CNS. Hence, at the present time it seems equally likely that, in the CNS, neuronal survival upon proper contact with the target area is regulated by a large number of different, population-specific neurotrophic factors, or alternatively, that there is only a small number of neurotrophic factors, which may be precisely regulated during development.

In an attempt to clarify the degree of selectivity of the actions of various neurotrophic substances, we used previously established cell culture models of fetal rat septum, pons, and ventral mesencephalon (Hartikka and Hefti, 1988a, b; Knusel and Hefti, 1988; Michel et al., 1989). Septal cholinergic cells in culture respond to NGF in various ways, among them by increasing the activity of the enzyme choline acetyltransferase (ChAT; Hefti et al., 1985; Hartikka and Hefti, 1988a, b; Hatanaka et al., 1988). Pontine cholinergic neurons, located in the pedunculopontine and dorsolateral tegmental nuclei, share some of the morphological characteristics of basal forebrain cholinergic neurons and have medium- to large-sized cell bodies and long centrally ascending axons (Woolf and Butcher, 1986; Rye et al., 1987; Goldsmith and van der Kooy, 1988). However, this cell group has been shown not to respond to NGF *in vitro* (Knusel and Hefti, 1988) and to lack receptors for NGF (Woolf et al., 1989). Cultures of the ventral mesencephalon were used to test for effects of trophic factors on dopaminergic cells of the substantia nigra. We have previously reported that CNTF does not seem to promote the differentiation of septal or pontine cholinergic neurons (Knusel and Hefti, 1988). In the present study trophic effects on septal and pontine cholinergic and mesencephalic dopaminergic neurons were examined for bFGF, insulin, IGF-I, IGF-II, EGF, and NGF. Further experiments were aimed at defining differences and similarities in the actions of NGF, bFGF, and insulin on septal cholinergic cells.

Materials and Methods

Preparation of the cultures. Rat fetuses of the embryonic age E15–E17 were collected into PBS from anesthetized (Nembutal, 0.6 ml/animal) mothers. The brains of the embryos were removed in L-15 medium without serum, and septal, mesencephalic, and pontine areas were dissected using previously published procedures (Hartikka and Hefti, 1988a; Knusel and Hefti, 1988; Michel et al., 1989). The tissue pieces were washed 2 times in medium and then mechanically dissociated by gently pipetting 20–30 times through a blue (1 ml) sterile pipet in about 1.5–2 ml of medium. Immediately after this trituration, approximately 10 ml of medium was added and the undispersed tissue pieces were allowed to settle during the 10–20 min. Most of the supernatant, which contained the individual cells, was then transferred to a second tube. About 1.5 ml medium was left in the tube for a second trituration. Virtually all of the tissue was dispersed after the second trituration. The cells were spun down in a centrifuge at 200 × *g* (10 min), resuspended in 5 ml of L-15 medium, and counted in a hemocytometer using exclusion of trypan blue as criterion for viability. Aliquots of 0.6–0.8 million viable cells were pipetted into wells of 16 mm diameter in 24-well plates (Costar) which contained 0.5 ml of growth medium (see below). Culture wells were previously coated overnight with a solution of 1 mg/ml polyethylenimine in 0.15 M sodium borate buffer, pH 8.3. The wells were washed 2–3 times with sterile PBS before medium was added.

The neurons were grown in Leibovitz's L-15 medium (purchased from Gibco) with the following additions: 5% horse serum, 0.5% fetal bovine serum, and per liter of medium 2.5 gm $NaHCO_3$, 9.0 gm glucose, 875 mg glutamine, 5 mg beta-alanine, 15 mg aspartic acid, 15 mg cystine, 15 mg glutamic acid, 5 mg para-aminobenzoic acid, 50 mg ascorbic acid, 10 mg choline chloride, 25 mg fumaric acid, 2.5 mg glutathione, 60 mg imidiazole, 10 mg myo-inositol, 0.5 mg alpha-lipoic acid, 2 mg vitamin B_{12} and 100,00 units penicillin G and 100 mg streptomycin. Cells were incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere. The medium was changed at approximately 3 hr and 24 hr after plating and, subsequently, every 2–3 d. Each change included 2 rinses of the cells with medium. Growth factors were added after each medium change as specified in the Results. The cells were taken for biochemical determination of ChAT activity or dopamine uptake after 6–8 d *in vitro*, for ChAT immunocytochemistry after 10–14 d.

ChAT and neurofilament immunocytochemistry. Cultures were washed 3 times with PBS, fixed for 30 min in fresh 4% paraformaldehyde in PBS at room temperature, and then incubated for 3–5 d at 4°C with a monoclonal antibody against ChAT (gift of Dr. F. Eckenstein, Oregon Health Science University) or against neurofilament (RT97, Wood and Anderton, 1981; a gift from Dr. J. Wood, Wellcome Research Laboratories, Beckenham, England). The antibody was contained in 0.1 M sodium phosphate buffer, pH 7.4, with 5% sucrose, 5% bovine serum albumin, and 0.1% Triton X-100 (PS- solution) and normal rabbit serum (1:100 dilution). Subsequent incubations at room temperature were with biotinylated anti-rat antibody (10 μg/ml PS, Vector Laboratories, Burlingame, CA) and then with an avidin-biotin conjugate of peroxidase (Vectastain), each for 2 hr. The peroxidase was visualized with diaminobenzidine and hydrogen peroxide.

Determination of ChAT activity and protein content. Cultures were washed with PBS and then homogenized by sonication in 250 μl of 50 mM Tris-HCl buffer, pH 6.0, with 0.3% Triton X-100. Aliquots (30–50 μl) of the homogenate were taken for the determination of ChAT activity according to the method of Fonnum (1975). [1-^{14}C] acetyl-coenzyme A (NEN) was diluted with unlabeled acetyl-CoA to give a final substrate

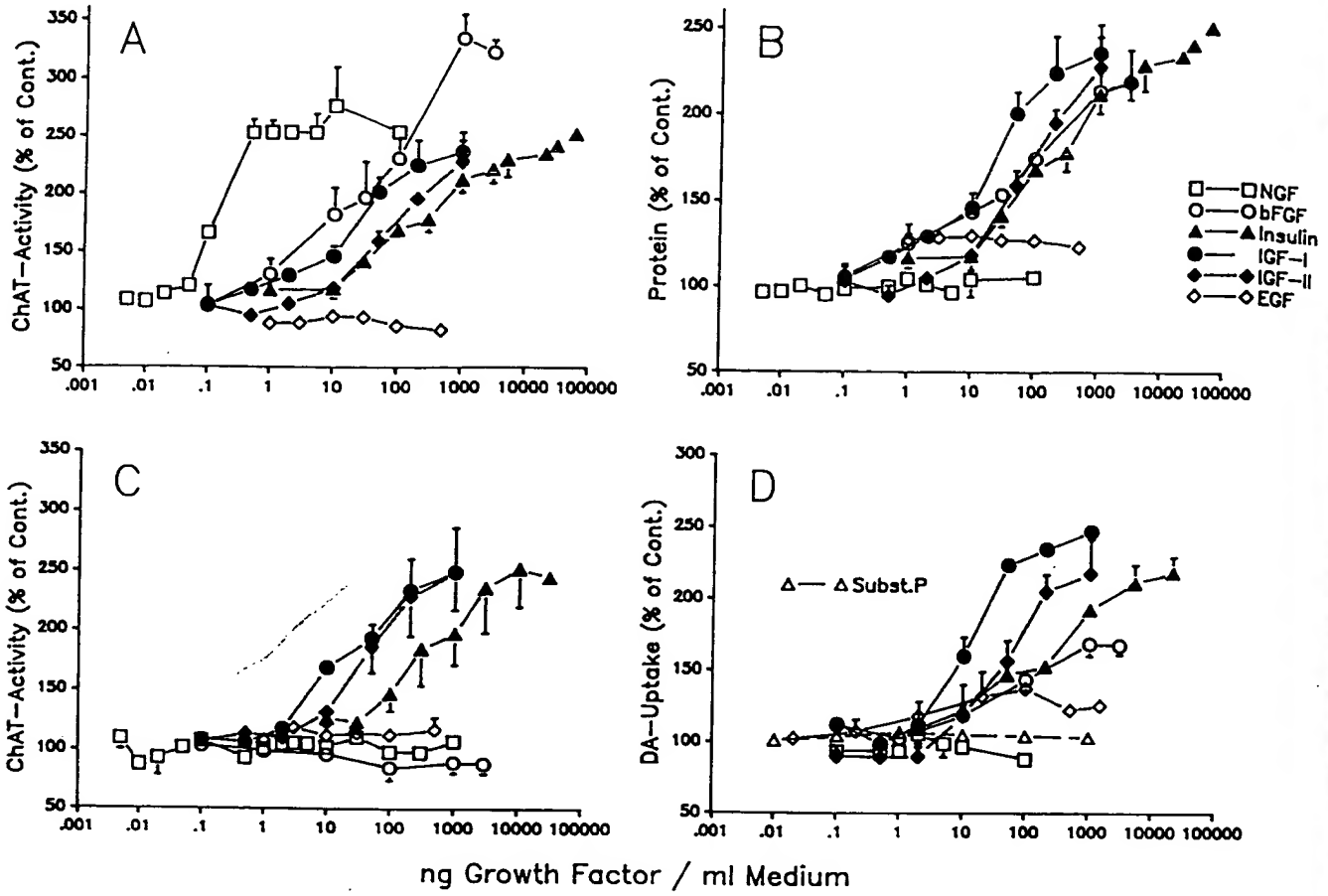

Figure 1. Dose-response curves for effects of growth factors on rat brain cultures. Cultures of dissociated fetal rat brain cells (E16/E17) were grown for 1 week in the presence of growth factors as indicated in the graphs. Plating density was 0.8 × 10^6/16-mm well. For each factor data were pooled from 2 or 3 experiments. Each symbol represents the mean of 4–8 individual cultures. Bars represent SEM; they were omitted where they would have appeared smaller than the symbol. Note order of potency in all 4 graphs: IGF-I > IGF-II > insulin. *A*, ChAT activity per well in septal cultures. Note high potency of NGF, but absence of effect in *B*, *C*, and *D*. *B*, Protein content per well in septal cultures. *C*, ChAT activity per well in cultures of pons. *D*, Dopamine uptake per well in cultures of ventral mesencephalon. Note limited effects of bFGF and EGF and absence of effect of substance P.

concentration of 20 μM (specific activity 4.09 Ci/mol of acetyl-CoA). Incubation was at 37°C for 20 min. Protein content of the cultures was measured according to the method of Bradford (1976) using bovine gamma-globulin (Bio-Rad) as a standard.

Dopamine uptake. The method used was a modification of that described by Prochiantz et al. (1981). Cells were washed twice with incubation solution (5 mM glucose, 1 mM ascorbic acid in PBS) and then preincubated for 5 min at 37°C with 250 μl incubation solution containing 1 mM pargyline. [^{3}H]dopamine (37 Ci/mmol) was then added to give a final concentration of 50 nM and the cultures were incubated at 37°C for 15 min. Blanks were obtained by incubating cells at 0°C. The uptake was stopped by removing the incubation mixture, immediately followed by 5 washes with cold PBS. The cells were then lysed in 1% Triton X-100 with 10% perchloric acid and transferred to scintillation vials for counting.

Materials. Chemicals of analytical grade were purchased from Sigma, if not otherwise stated. Media and sera for tissue cultures were obtained from Gibco. Mouse NGF was purified according to Suda et al. (1978). A polyclonal sheep anti-NGF serum (Suda et al., 1978) was used, 1 μl/ml of which inhibited the biological activity of up to 2 μg/ml of NGF. Bovine insulin and mouse submaxillary gland EGF were purchased from Sigma. Human recombinant bFGF was provided by Synergen (Boulder, CO). This molecule was shown to stimulate proliferation of various cells at concentrations of 1–10 ng/ml. bFGF was stored at 2 mg/ml in 10 mM sodium phosphate buffer, pH 7.0, containing 0.3 M glycerol and 0.3 mg/ml heparin. This stock solution was kept at −70°C and further dilutions were prepared immediately before addition to the cultures. Human recombinant IGF-I and IGF-II were obtained from Eli Lilly Laboratories (Indianapolis, IN). Stock solutions of 1 mg/ml were kept at −70°C and further diluted in growth medium.

Results

Trophic actions on septal cholinergic neurons

In septal cultures, the activity of the cholinergic marker enzyme, ChAT, was elevated by bFGF, insulin, IGF-I, and IGF-II in a similar way as earlier shown for NGF. The dose-response relationship was established for each of the compounds (Fig. 1*A*). NGF was most potent in stimulating ChAT activity, producing a half-maximal response at a concentration of 0.16 ng/ml medium (6.0 pM), whereas these concentrations were 88.8 ng/ml (5.4 nM) for bFGF, 255.0 ng/ml (42.5 nM) for insulin, 24.4 ng/ml (3.19 nM) for IGF-I, and 76.4 ng/ml (10.2 nM) for IGF-II as determined from the pooled data of 2–3 experiments per growth factor (Fig. 1*A*). When measured 7 d after plating, the various factors were approximately equally effective in producing elevation of ChAT activity, but concentrations of approximately 1 ng/ml NGF (38 pM), 1 μg/ml IGF-I or IGF-II (130 nM) or bFGF (60 nM), or 5 μg/ml insulin (800 nM) had to be used to produce maximal responses. EGF at concentrations between 1

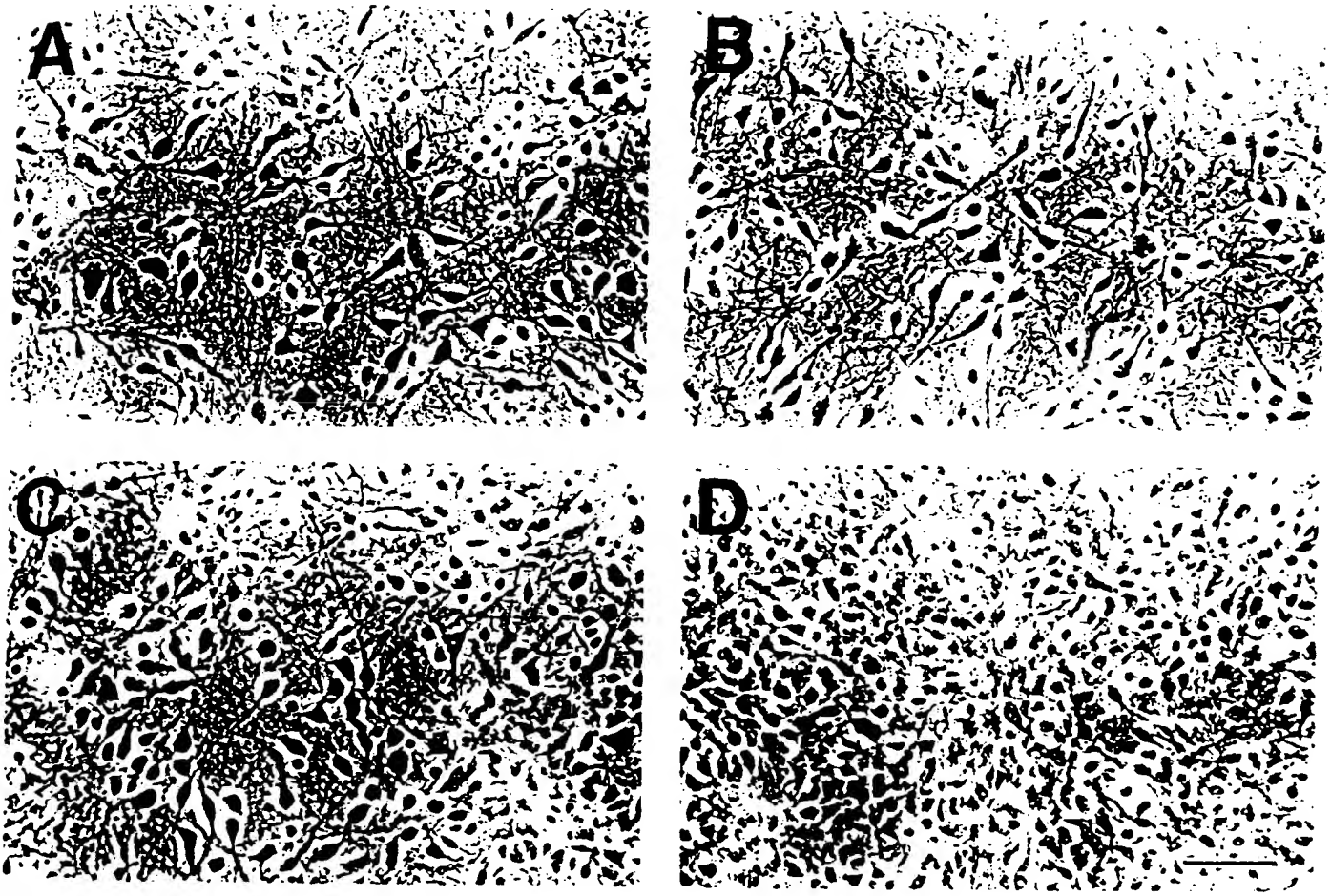

Figure 2. Phase-contrast micrographs of living septal cultures grown in presence of NGF, insulin, or bFGF. Cells were grown for 7 d without growth factors (*A*), in the presence of 50 ng/ml NGF (*B*), 30 μg/ml insulin (*C*), or 1 μg/ml bFGF (*D*). Plating density was 0.8 × 10^6 cells/well. Control cultures and cultures grown with NGF appear identical whereas the presence of insulin or bFGF substantially increased cell density. Scale bar, 50 μm.

and 500 ng/ml (0.17 and 82 nM) failed to increase ChAT activity in septal cultures (Fig. 1*A*). Similarly, no effects were observed with PDGF, which was tested between 0.6 and 15 ng/ml (data not shown).

Trophic actions on pontine cholinergic neurons and mesencephalic dopaminergic neurons

As shown previously (Knusel and Hefti, 1988) and in marked contrast to the situation in septal cultures, NGF in concentrations up to 1000 ng/ml did not enhance ChAT activity in pontine cultures (Fig. 1*C*). NGF also failed to elevate dopamine uptake in mesencephalic cultures (Fig. 1*D*). In contrast, insulin, IGF-I, and IGF-II markedly increased transmitter-specific differentiation in both systems. ChAT activity in pontine cultures and dopamine uptake in mesencephalic cultures were elevated by 100–200% above control levels (Fig. 1, *C*, *D*). Effective concentrations of the 3 factors in pontine and mesencephalic cultures were similar to those observed in septal cultures. ED_{50}s were calculated in pontine cultures at 386.4 ng/ml for insulin (64.4 nM), 21.8 ng/ml for IGF-I (2.8 nM), and 42.6 ng/ml for IGF-II (5.7 nM) and in mesencephalic cultures at 311.5 ng/ml for insulin (51.9 nM), 13.9 ng/ml for IGF-I (1.8 nM), and 47.5 ng/ml for IGF-II (6.4 nM). The rank order of potency, therefore, in all 3 culture systems was IGF-I > IGF-II > insulin. bFGF, in concentrations between 10 and 3000 ng/ml, did not enhance ChAT activity in pontine cultures but elevated dopamine uptake in mesencephalic cultures, although to a smaller degree than the insulin family of growth factors (Fig. 1, *C*, *D*). As in septal cultures, EGF at concentrations between 1 and 500 ng/ml failed to increase ChAT activity in pedunculopontine cultures (Fig. 1*C*). In mesencephalic cultures these concentrations resulted in a modest maximal increase of dopamine uptake of 38% above control levels (Fig. 1*D*). Half-maximal response in mesencephalic cultures was achieved with 61.8 ng/ml bFGF (3.7 nM) and 1.0 ng/ml EGF (0.16 nM). Substance P was recently reported to elevate the activity of tyrosine hydroxylase in cultures of substantia nigra cells (Friedman et al., 1988). Addition of 0.1 ng to 10 μg/ml (74 pM and 7.4 μM) of substance P did not influence dopamine uptake in our mesencephalic cultures (Fig. 1*D*).

Mitogenic effects

bFGF, which is a potent mitogen for various cell populations, stimulated cell proliferation in our cultures. Three to 4 d after plating, the density of cells was clearly higher than in control cultures, and usually after 5 d *in vitro*, the cultures were confluent. After about 1 week *in vitro* cultures treated with bFGF showed a very high rate of metabolism requiring daily change

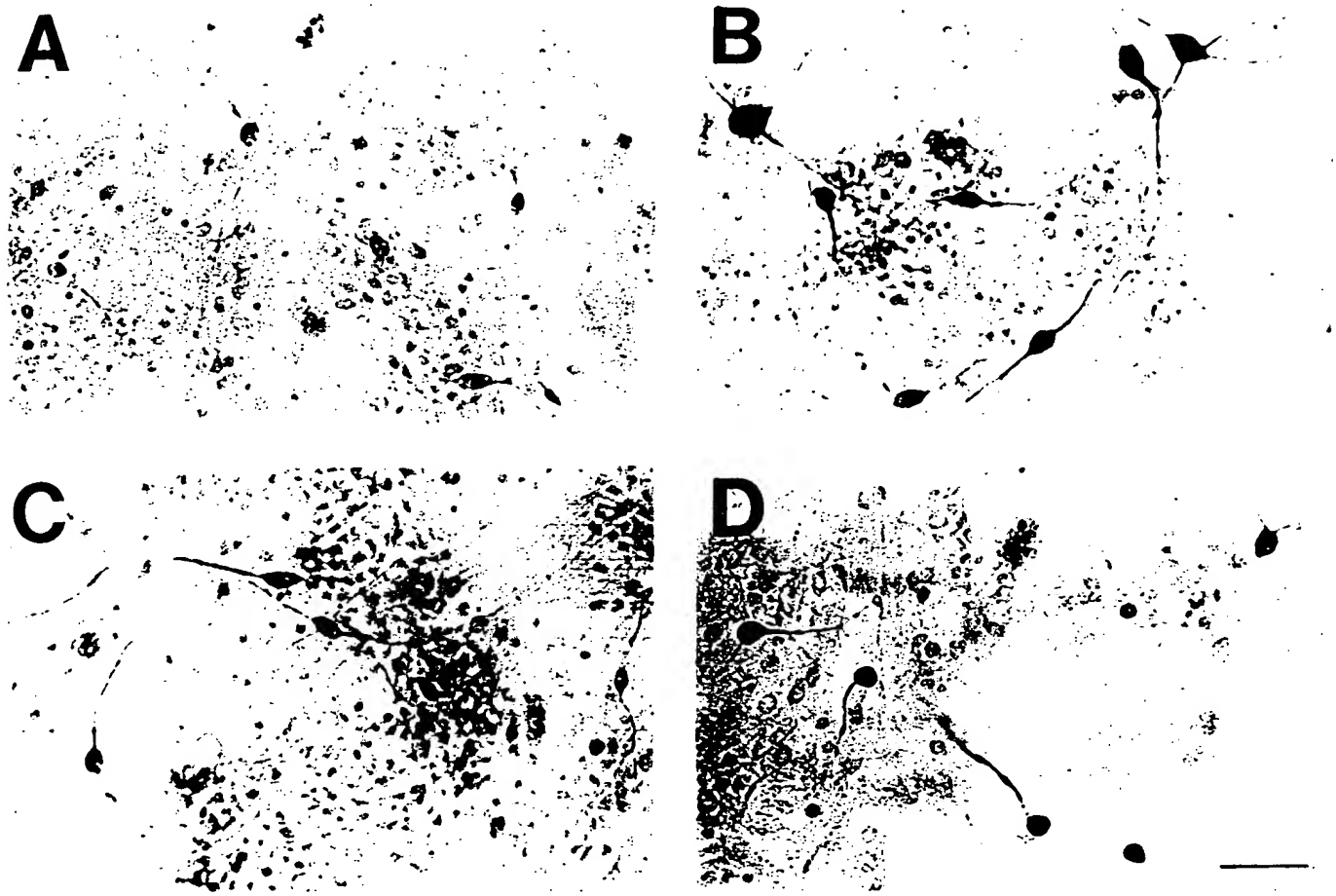

Figure 3. Bright-field micrographs of septal cultures immunostained with a monoclonal antibody against ChAT. Cultures were grown for 10 d. Plating density and culture conditions were the same as those of the cultures shown in Figure 2. *A*, No growth factors; *B*, 50 ng/ml NGF; *C*, 30 μg/ml insulin; *D*, 1 μg/ml bFGF. Note the weak staining intensity in *A*, intermediate intensity in *C*, and strong staining in *B* and *D*. Cell bodies in bFGF-treated cultures (*D*) appear smaller than those treated with NGF (*B*). Scale bar, 50μm.

of medium to avoid acidification. At this time, bFGF-treated cultures were densely packed and their appearance in phase-contrast microscopy was clearly different from that of control or NGF-treated cultures (Fig. 2). In agreement with the visual observations, bFGF produced a dose-dependent increase in protein mass in all 3 culture systems used. This finding for septal cultures is shown in Figure 1*B*. The presence of insulin, IGF-I, or IGF-II resulted in cell proliferation and a similar maximal protein increase as bFGF (Figs. 1*B*, 2*C*). The cultures, however, as judged from the acidification of the medium, did not develop the same high rate of metabolism. EGF increased protein content at concentrations from 1 to 500 ng/ml, but the increase in protein content was less than was attained with bFGF, insulin, or the insulin-like growth factors. NGF, as shown previously (Hartikka and Hefti, 1988a; Knusel and Hefti, 1988), did not influence the protein content in septal, pontine, or mesencephalic cells. Similarly, PDGF failed to elevate the protein content of septal cultures (data not shown). Growth factors that stimulated both transmitter specific differentiation and cellular proliferation always showed similar concentration requirements for these effects (Fig. 1, *A*, *B*).

Table 1. Effects of insulin, IGF-I, and IGF-II on septal cultures

Growth factors	ChAT/well (pmol/min)	Protein/well (μg)
CONT	82.6 ± 2.9	146.2 ± 3.6
IGF-I	167.6 ± 4.1	282.7 ± 10.9
IGF-II	153.0 ± 9.1	251.8 ± 5.4
Insulin	149.0 ± 3.0	253.1 ± 2.9
IGF-I + IGF-II	163.3 ± 6.4	278.9 ± 6.0
IGF-I + Insulin	153.1 ± 7.0	264.0 ± 7.6
IGF-II + Insulin	159.3 ± 8.3	258.0 ± 13.5

Cultures were prepared and grown like those shown in Figure 2; concentrations: IGF-I and IGF-II, 1 μg/ml; insulin, 30 μg/ml. Values are means ± SEM; $n = 4$–8. All differences with control are statistically significant ($p < 0.001$; Student's *t*-test).

Further characterization of action of bFGF and insulin on septal cholinergic neurons

The actions of IGF-I, IGF-II, and insulin on septal cultures were not additive. Combining maximally effective concentrations of these substances did not elevate ChAT activity or protein content above the level attained by either of the 3 factors alone (Table 1). Given these findings, further studies were limited to insulin, which served as a representative for this family of growth factors.

Biochemical determination of ChAT activity of neuronal cul-

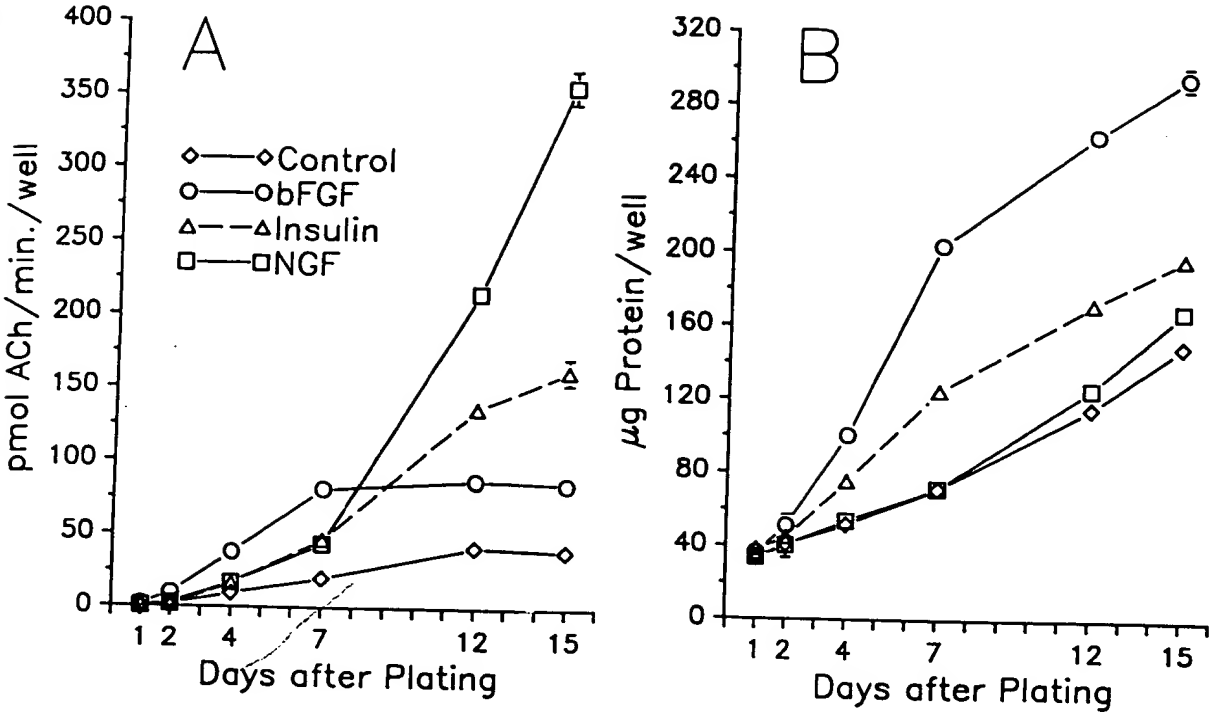

Figure 4. Time course of action of NGF, bFGF, and insulin on ChAT activity and protein content in septal cultures. At day 0, 0.6 × 10^6 fetal (E16) rat septal cells were plated per 16-mm well. Growth factors were added immediately after plating and with every medium change. Four cultures per treatment group were taken for ChAT and protein assays at the indicated times. The rate of increase of ChAT activity was initially highest in bFGF-treated cultures but higher final levels were reached with insulin or NGF treatment.

tures does not discriminate between increased survival of cholinergic cells and increased expression of the enzyme in individual cells. To tentatively assess the presence of the enzyme in individual cells, septal control cultures and cultures treated with NGF, insulin, or bFGF were stained immunocytochemically for ChAT. As shown earlier (Hartikka and Hefti, 1988a; Knusel and Hefti, 1988), only a small number of weakly stained ChAT-positive neurons was visible in control cultures (Fig. 3*A*), whereas in NGF-treated septal cultures, ChAT staining revealed many darkly stained, large bipolar and multipolar cells with branched processes (Fig. 3*B*). Similarly, addition of bFGF and insulin to the medium elevated staining intensity of ChAT-positive neurons. The staining intensity in bFGF-treated cultures was comparable to that in NGF-treated cultures but the cell bodies seemed to be smaller and to carry fewer branched processes (Fig. 3*D*). Slightly lower staining intensities were typical of cultures grown in presence of insulin but the appearance of the neurons was similar to that in NGF-treated cultures (Fig. 3*B*), except for their smaller cell bodies. Treatment with either of the 3 factors increased staining intensity of maximally ChAT-positive neurons above the maximal staining observed in control cultures. Although immunocytochemical staining intensity might not directly reflect the concentration of antigen, these observations suggest that NGF, bFGF, and insulin elevate ChAT expression in individual cholinergic neurons. Further evidence that bFGF most likely increases ChAT activity in individual neurons was provided by an experiment that employed different plating densities (data not shown). bFGF was equally effective in low density (0.2 Mio. cells/16-mm well) as in high-density cultures (0.8–3.2 Mio cells/16-mm well), where survival of cholinergic neurons is maximal and cannot be further increased by NGF (Hartikka and Hefti, 1988a).

Despite the similarities of the effects of bFGF, insulin, and NGF on cultured septal cholinergic neurons, there were pronounced differences among these factors with regard to the time course of their action on ChAT activity (Fig. 4). During the first week *in vitro* ChAT activity was highest in bFGF-treated cultures (Fig. 4*A*). After 7 d bFGF produced no further increase in ChAT activity but continued to stimulate proliferation of non-neuronal cells as reflected by a continuous rise of the protein content (Fig. 4*B*). In contrast, the most pronounced increase of ChAT activity in NGF-treated cultures was observed after more than 7 d in culture. Similarly, in the presence of insulin the ChAT increase was more pronounced after 7 d *in vitro* but, in contrast to NGF-treated cultures, started to level off after approximately 12 d *in vitro*. Final levels of ChAT activity which were measured after 15 d *in vitro* were 909% of control in NGF-treated cultures, 413% in cultures treated with insulin, and 216% in cultures treated with bFGF.

To test whether septal cholinergic neurons are more responsive to bFGF early *in vitro*, or if the lower final level of ChAT activity in bFGF-treated cultures in Figure 4 simply reflects an inability of our culture conditions to support the excessive growth of these cultures, the 3 factors were applied at different times after plating (Fig. 5). Each set of cultures was grown in the presence of the factors for 3 d and then taken for ChAT assay. When the factors were given immediately after plating, ChAT activity was increased to 327% of control by bFGF, to 184% by NGF, and to 125% by insulin. If the factors were added after the cultures had been grown for 8 d under control conditions, the relative effects were 110% for bFGF, 266% for NGF, and 131% for insulin. This result confirms a pronounced effect of bFGF at early culture times. At late times bFGF is less effective, whereas for NGF an inverse relationship holds true. In contrast,

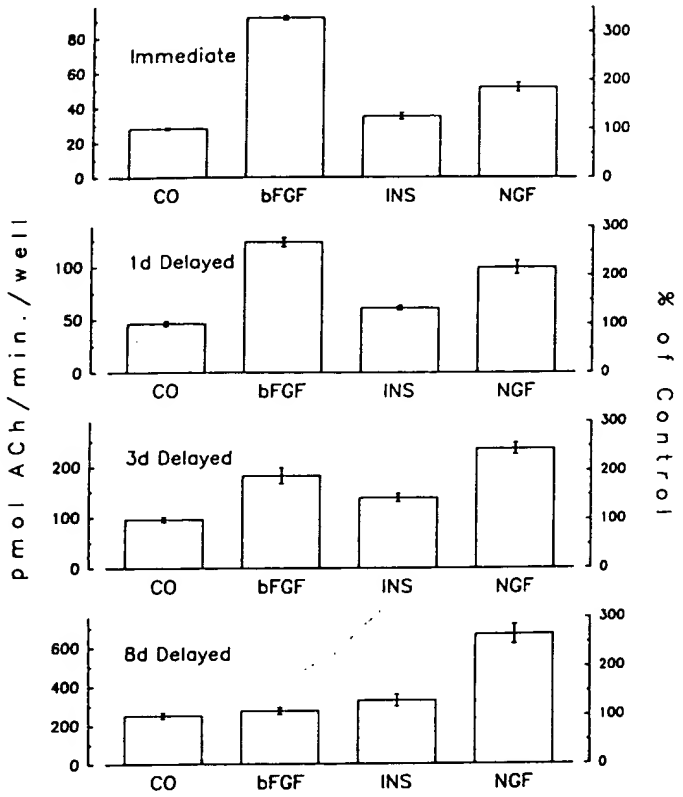

Figure 5. Change of responsiveness of septal cultures to NGF, bFGF, and insulin during culture time. Growth factors were added to cultures of fetal rat septal cells at different times after plating. The cultures were then grown for 3 additional days in the presence of the factors and taken for the measurement at the end of this 3-d treatment period. Note the high ChAT increase induced by bFGF at early and by NGF at late *in vitro* times. bFGF: 1 μg/ml bFGF; INS: 30 μg/ml insulin; NGF: 50 ng/ml NGF.

insulin increases ChAT activity about equally at all times. The low response to bFGF after 8 d and to insulin does not reflect a principal inability of the cultures to respond. If the cultures were grown in presence of growth factors for 6 d instead of 3 after a delay of 8 d, bFGF increased ChAT activity to 166%, insulin to 165%, and NGF to 574% of control levels (data not shown). The large effect of bFGF on ChAT activity at initial culture times could reflect enhanced survival of freshly dissociated neurons during the first days in culture, as shown for various neuronal populations in serum-free medium (Morrison et al., 1986; Walicke et al., 1986; Walicke, 1988), or increased mitosis of neuronal precursors (Gensburger et al., 1987). We therefore counted the total number of neurons present in our cultures after 2 d *in vitro* and compared the results with determinations of ChAT activity in cultures of the same age. Neurons were identified using neurofilament immunocytochemistry. Cultures treated with bFGF did not contain significantly more neurons than NGF- or insulin-treated cultures or untreated controls after 2 d *in vitro* (Table 2), whereas ChAT activity was elevated to 335% of control with bFGF, 151% of control with NGF, and 120% of control with insulin treatment (Table 3). These findings suggest that the early bFGF-mediated elevation of ChAT activity is not due to a general promotion of neuronal survival or stimulation of neuroblast mitosis in our cultures. They do not rule out the possibility of a selective enhancement of the survival of cholinergic neurons.

Since bFGF and insulin strongly promoted the growth of non-neuronal cells and may affect other neuronal populations besides the cholinergic cells in our septal cultures, their stimulatory actions on these neurons could be indirect and secondary to an action on receptors located on other cells. In particular, in septal cultures bFGF or insulin could stimulate production and release of NGF. Anti-NGF antibodies, at a sufficient concentration to completely block the NGF-mediated increase of ChAT activity, failed to abolish the bFGF- or insulin-mediated elevations (Fig. 6). This experiment does not completely rule out the possibility that the antibodies failed to penetrate to the sites of synthesis and action of endogenously produced NGF. However, the effects of maximally effective concentrations of bFGF, insulin, and NGF on ChAT activity and protein content were additive (Table 4), suggesting that NGF, bFGF, and insulin each stimulate different cellular mechanisms.

To test whether the effects of bFGF and insulin on cultured cholinergic neurons were mediated by glial cells, cell proliferation was inhibited by the addition of cytosine arabinoside (ara-C, 1.8 μM) to the medium (Table 5). We have earlier shown that adding ara-C to our septal cultures reduces the number of astrocytes to less than 5% of the number counted in control cultures without affecting the number of neurons (Hartikka and Hefti, 1988a). In confirmation of our earlier results, NGF was found to elevate ChAT activity in septal cultures in presence or absence of glial cells. Similarly, bFGF produced similar relative increases in ChAT activity in septal cultures, and insulin in septal, pontine, and mesencephalic cultures with or without ara-C, suggesting that the action of neither factor in these cultures depends on the presence of glial cells. In contrast, in mesencephalic cultures, the stimulatory action of bFGF and EGF on dopamine uptake was abolished by the presence of ara-C in the medium.

Table 2. bFGF, insulin, and NGF do not enhance cell survival during initial 48-hr period *in vitro*

Growth factors	Surviving cells
Control	386,643 ± 19,014
bFGF (1 μg/ml)	405,215 ± 18,468
Insulin (30 μg/ml)	406,143 ± 9959
NGF (50 ng/ml)	396,805 ± 11,726

Cultures prepared like those shown in Figure 2 were fixed after 2 d *in vitro* and taken for immunocytochemical visualization of neurofilament proteins. Means ± SEM; $n = 12$.

Table 3. bFGF but not insulin or NGF produces a profound elevation of ChAT activity in septal cultures during the initial 48-hr period *in vitro*

Growth factors	ChAT/well (pmol/min)
Control	9.9 ± 0.7
bFGF (1 μg/ml)	33.2 ± 1.8[a]
Insulin (30 μg/ml)	11.9 ± 0.7
NGF (50 ng/ml)	15.0 ± 0.5[b]

Cultures were grown like those shown in Figure 2 but taken for analysis after only 2 d *in vitro*. Values given are means ± SEM; $n = 6$.

[a] Higher than control value and value of NGF group, $p < 0.001$.

[b] Higher than control value, $p < 0.002$ (Student's t-test).

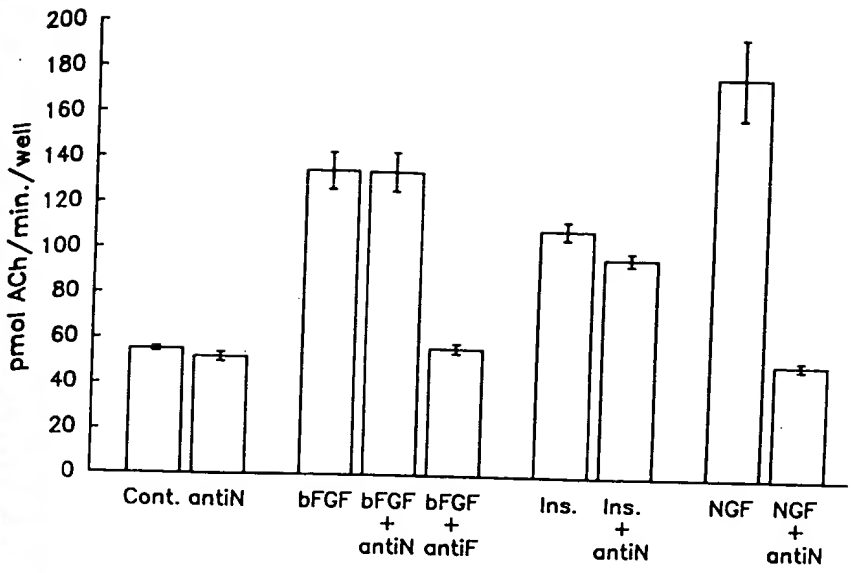

Figure 6. NGF- and bFGF-specific immunological blockage of ChAT increase in septal cultures. Cultures were grown as for Figures 2 and 3. The NGF-mediated ChAT increase, but not the bFGF- or insulin-mediated increase, was completely blocked by antiserum against NGF. Monoclonal antibodies against bFGF prevented the bFGF-mediated ChAT increase.

Discussion

It is well established that developmental growth and differentiation of septal forebrain cholinergic neurons is stimulated by NGF (Honegger and Lenoir, 1982; Gnahn et al., 1983; Hefti et al., 1985; Hatanaka and Tsukui, 1986; Mobley et al., 1986; Johnston et al., 1987; Martinez et al., 1987; Hartikka and Hefti, 1988a). The present study suggests that bFGF, insulin, and the insulin-like growth factors, but not EGF, exert similar actions. The actions of NGF, bFGF, and insulin on septal cholinergic neurons were additive and appear to be mediated by different mechanisms, whereas insulin, IGF-I, and IGF-II probably act via the same receptors. Development of pontine cholinergic neurons *in vitro* was stimulated by insulin and IGFs, but not by NGF, bFGF, or EGF. *In vitro* development of mesencephalic dopaminergic neurons was stimulated by insulin, IGFs, and, to a smaller degree, by bFGF and EGF. In all 3 culture systems, proliferation of non-neuronal cells was increased by insulin, IGFs, bFGF, and EGF, but not NGF.

bFGF, which was originally purified from brain and pituitary based on its mitogenic activity for Balb/c 3T3 cells (Gospodarowicz et al., 1978), is a potent mitogen for many cells (see Gospodarowicz, 1984). Recently, this molecule has been shown *in vitro* to support survival and neurite outgrowth of neurons from various brain regions (Morrison et al., 1986; Walicke et al., 1986; Unsicker et al., 1987; Hatten et al., 1988; Walicke, 1988) and to promote neuronal differentiation of PC12 cells (Rydel and Greene, 1987; Schubert et al., 1987). Intracerebral administration of bFGF prevents degenerative changes of lesioned cholinergic neurons of the basal forebrain (Anderson et al., 1988; Otto et al., 1989). Although bFGF stimulates proliferation and influences the morphology of astrocytes in culture (Kniss and Burry, 1988; Perraud et al., 1988), its actions on neurons are believed not to be mediated by glial cells (Morrison et al., 1986; Walicke and Baird, 1988). Several observations make it seem likely that the effects of bFGF on the cholinergic neurons in our septal cultures resulted from a direct action of this molecule on the cholinergic cells. First, bFGF was most effective at very early culture times (1–2 d *in vitro*) when cell-cell contact is still minimal. Second, preventing proliferation of

Table 4. Additivity of the increases in ChAT activity produced by bFGF, insulin, and NGF in septal cultures

Growth factors	ChAT/well (pmol/min)	Protein/well (μg)
CONT	20.2 ± 0.3	76.2 ± 4.9
bFGF	65.0 ∓ 4.1	212.6 ± 15.1
Insulin	47.1 ± 8.6	162.6 ± 22.0
NGF	43.3 ± 7.4	70.6 ± 3.7
NGF + bFGF	85.6 ± 12.0[a]	194.9 ± 25.8
NGF + insulin	97.9 ± 6..5[a]	149.0 ± 7.4
bFGF + insulin	83.5 ± 3.1[a]	348.7 ± 8.2[a]
NGF + bFGF + insulin	117.6 ± 2.2[a,b]	348.1 ± 12.2[a]

Cultures were grown for 6 d. Means ± SEM; $n = 4$; concentrations factors: NGF, 50 ng/ml; bFGF, 1 μg/ml; insulin, 30 μg/ml. Values of all treated groups are higher than those of control group, $p < 0.01$.

[a] Higher than value obtained in culture grown with one of the growth factors only, $p < 0.05$.

[b] Higher than group treated with bFGF and insulin, $p < 0.05$.

Table 5. Preventing cell proliferation abolishes effects of bFGF and EGF in mesencephalic cultures but does not prevent the action of bFGF and NGF in septal cultures and of insulin in all culture types

		No Ara-C	Ara-C (1.8 μM)
Septal cultures (pmol ACh/min/well)	Control	114.4 ± 7.5	82.1 ± 7.3
	bFGF	251.3 ± 26.4[b]	217.4 ± 17.7[c]
	Insulin	180.9 ± 20.4[a]	120.4 ± 12.9[a]
	NGF	370.0 ± 28.6[c]	179.1 ± 18.1[b]
Pontine cultures (pmol ACh/min/well)	Control	17.2 ± 0.2	20.1 ± 1.3
	bFGF	14.7 ± 0.5[b]	19.9 ± 0.7
	Insulin	32.6 ± 2.2[c]	35.8 ± 1.9[c]
Mesencephalic cultures (fmol DA/min/well)	Control	55.3 ± 2.3	57.5 ± 2.7
	bFGF	86.4 ± 5.9[b]	60.7 ± 4.2
	Insulin	106.3 ± 8.3[b]	92.2 ± 5.7[b]
	EGF	70.0 ± 5.0[a]	58.6 ± 3.3

Cultures were grown for 8 d. Means ± SEM; $n = 4$–6; concentrations of factors as in Table 4; [a,b,c] significantly higher than corresponding control.

[a] $p < 0.05$.

[b] $p < 0.01$.

[c] $p < 0.001$ (Student's t-test).

cells by adding ara-C did not diminish the actions of bFGF. Third, transfer of medium from cultures grown in presence of bFGF did not reveal any "conditioning" effect of bFGF (data not shown). The bFGF-mediated elevation in ChAT activity, at least at early times *in vitro* when this effect is particularly pronounced, does not reflect a general effect of bFGF on survival of dissociated neurons in culture as could be expected from the above-mentioned data (Morrison et al., 1986; Walicke et al., 1986) or, alternatively, a general proliferation of neuronal precursors (Gensburger et al., 1987). Under our culture conditions, bFGF did not increase the total number of cells in septal cultures up to 2 d after plating. However, we cannot exclude the possibility that bFGF could have selectively promoted the survival of cholinergic neurons.

The concentrations of bFGF required to stimulate cholinergic differentiation in our septal cultures were higher than those found to promote survival and neurite outgrowth of freshly plated neurons. Reported concentrations producing 50% of maximal neuron survival range from 15 pg/ml to 1 ng/ml (Morrison et al., 1986; Walicke et al., 1986; Unsicker et al., 1987; Walicke, 1988). Concentrations of bFGF stimulating half-maximal proliferation of various non-neuronal cells have been found to be approximately 50 pg/ml (Esch et al., 1985; Ferrara et al., 1988). Walicke et al. (1989) recently characterized a neuronal bFGF receptor with an affinity 10 times lower than that of mesenchymal bFGF receptors, suggesting that neuronal cells respond to higher concentrations of bFGF. We observed some variation among dose-response curves for bFGF in our experiments with septal cultures. The lowest concentration that was half-maximally active in any of the experiments was 21.4 ng/ml. Since determination of bFGF by radioimmunoassay in our solutions revealed a loss of about half of the bFGF during routine handling of the substance (data not shown), the actual minimal concentration of bFGF in the medium producing a half-maximal response probably was approximately 10 ng/ml. Still, the exact reason for the higher dose requirements in our cultures remains to be determined. Possibly, at least part of the difference might be due to differences in plating densities of the cells. We plated 3–5 × 10^5/cm² in our experiments whereas only 16 × 10^3/cm² cells were plated in neuronal survival experiments (Walicke et al., 1986). Under our culture conditions the potency of bFGF seemed to be negatively correlated with plating density. It has indeed been shown for a nontransformed cell line that the number of surface bFGF receptors decreases with increasing cell density (Veomett et al., 1989). It also seems possible that the added bFGF is more rapidly removed from the medium at higher cell density by specific and nonspecific binding or other mechanisms and that the effective concentration of bFGF in the medium may decline very quickly.

Insulin, IGF-I, and IGF-II promoted transmitter-specific development in all 3 culture systems to a similar extent. Maximal concentrations of the 3 factors, when applied to septal cultures, were equally effective in increasing ChAT activity and combinations of the factors did not result in additive effects, suggesting that insulin, IGF-I, and IGF-II acted via the same receptors. The rank order of potency in all 3 systems, IGF-I > IGF-II > insulin, is in accordance with the relative affinities of the factors for a recently characterized neuronal IGF-I receptor (Burgess et al., 1987). Insulin, IGF-I, and IGF-II, and their mRNA and receptors have been reported to occur and to be heterogeneously distributed in the adult mammalian brain (Dorn et al., 1982; Sara et al., 1982; Hill et al., 1986; Mendelsohn, 1987; Bohannon et al., 1988; Rotwein et al., 1988). None of the areas used for our cell cultures or the target regions of the neuronal populations studied have been recognized as being rich in insulin or IGF receptors in the above-mentioned studies. Thus, it seems surprising that insulin, IGF-I, and IGF-II act similarly and profoundly on cholinergic neurons of the septal and pontine areas as well as on the dopaminergic neurons of the substantia nigra in culture. However, it has been shown that the expression of insulin and IGFs and of their receptors is developmentally regulated in a complex and pronounced way with generally high levels of mRNA and receptors for the IGFs (Bassas et al., 1985; Pomerance et al., 1988; Rotwein et al., 1988). The regional distributions of receptors for insulin and IGFs or mRNAs coding for receptor proteins in the embryonic brain are not known. It is conceivable that insulin and, in particular, the IGFs play a different, and more widespread role during early neuronal development than in the adult brain. This concept is supported by findings that insulin and IGFs can support neuronal survival and induce neurite formation and the synthesis of neural proteins in developing neurons (Bothwell, 1982; Bhat, 1983; Puro and Agardh, 1984; Recio-Pinto et al., 1986; Aizenman and DeVellis, 1987; Kyriakis et al., 1987, DiCicco-Bloom and Black, 1988).

Insulin and the IGFs are known to stimulate the synthesis of glial proteins in fetal rat brain cell cultures (Lenoir and Honegger, 1983), DNA, RNA, proteins in astroglial cell cultures (Avola et al., 1988), and lipids in oligodendrocyte-enriched glial cultures (Van der Pal et al., 1988). In our cultures, insulin and the IGFs increased cell density and protein content. However, treatment of the cultures with ara-C to prevent cellular proliferation only slightly diminished the transmitter-specific effects of insulin. It seems likely that insulin, and probably also IGF-I and IGF-II, elevated ChAT activity and dopamine uptake in our cultures by a direct action on neurons and, to a minor extent, by indirect action via stimulation of glial cell proliferation. While a direct action on cholinergic and dopaminergic neurons seems likely, our studies do not rule out the possibility that other neurons mediated these effects. Any indirect stimulations mediated by glial cells or neuronal cells do not involve NGF or bFGF, since, in septal cultures, the effect of insulin was additive to the action of either of the other factors and since antibodies against NGF failed to block the insulin effect.

Many recent studies on neurotrophic actions have been based on the concept of target-derived survival factors, as suggested by the well-known biology of NGF (for reviews, see Thoenen and Edgar, 1985; Purves, 1986; Thoenen et al., 1987; Barde, 1989). This concept does not exclude that a multitude of different factors are required at specific ontogenetic stages for a neuron to develop properly and to maintain its structural integrity and function during adult life. Different roles for bFGF, insulin, IGFs, and NGF in neural development are suggested by the observed time courses of their stimulatory action on ChAT activity in septal cultures. During the first 7 d *in vitro*, bFGF induced a significantly larger increase of ChAT activity than insulin or NGF. In the cultures with insulin, ChAT activity rose up to day 15 but the rate of increase declined after 12 d *in vitro*, whereas in cultures with NGF the most dramatic increase in ChAT activity occurred after 7 d. A second experiment in which bFGF, insulin, and NGF were applied at different times of the culture period confirmed a relatively higher responsiveness of the cholinergic neurons to bFGF at early and to NGF at late *in vitro* times (see Fig. 5). A reduced late effect of bFGF

could be explained by the presence of endogenous bFGF in the control cultures at later culture times. Still, even maximal concentrations of bFGF, present for 6 d after a delay of 1 week (data not shown) or present during an entire culture period of 15 d (see Fig. 4), resulted in a much lower level of ChAT activity than the presence of NGF under the same conditions.

An alternative interpretation to an intrinsic, timed modulation of responsiveness to bFGF is suggested by a possible role of bFGF in repair processes (Finklestein et al., 1988). The mechanical trituration of the tissue that is used to dissociate embryonic cells in the preparation of the cell cultures severs existing neurites and may also disrupt cell body membranes. During the initial culture period, stimulation of repair processes may lead to similar measurable effects as enhancement of developmental processes. It can be speculated that the relatively larger effect of bFGF during the initial culture period demonstrates an active role of this substance in healing and regeneration, whereas NGF, which exerts its most significant influence on ChAT activity during later stages, promotes differentiation of the cholinergic cells. Although attractive, this hypothesis is not supported by our finding that bFGF did not enhance ChAT activity in pontine cultures, where, probably because of the more advanced development, the dissociation inflicts more cell damage than in septal cells (Knusel and Hefti, 1988). Our finding that NGF, bFGF, and insulin increase the intensity of the immunocytochemical staining for ChAT suggests that the biochemically measured ChAT activity reflects elevated expression of this protein by cholinergic neurons. However, it does not rule out that bFGF and insulin promote survival of cholinergic neurons as earlier shown to occur with NGF under specific culture conditions (Hartikka and Hefti, 1988a). Furthermore, it cannot be excluded that the 3 growth factors may affect different subpopulations of cholinergic neurons or induce noncholinergic neurons to express cholinergic traits. Further studies will be needed to test for these possibilities.

We believe that part of the importance of the present study lies in the comparison of effects of several known trophic substances on different neuronal populations studied under identical conditions. The exact roles and importance of neurotrophic factors in CNS development are poorly understood. While there is strong evidence that NGF serves as a target-derived neurotrophic factor for cholinergic neurons of the basal forebrain and promotes their differentiation, it is still unclear whether, similar to the situation in the peripheral sympathetic system, NGF controls the developmental survival of these cells. No comparable target-derived neurotrophic factors for other central neuronal populations have been identified with certainty. It is not known whether most or all central neuronal populations depend on target-derived factors for development and survival and, if they do, whether there exists a multitude of population-specific factors or only a small number of factors with a precise developmental regulation of their mechanisms. Our results confirm the relative selectivity of the action of NGF on the basal forebrain cholinergic neurons despite recent reports of a more widespread localization of NGF and NGF receptors in developing than in adult brain (Buck et al., 1988; Ernfors et al., 1988; Schatteman et al., 1988; Yan and Johnson, 1988; Large et al., 1989). bFGF, similar to NGF, strongly stimulates ChAT activity in septal cholinergic neurons. Considering the anteroposterior gradient in brain development, a developmentally short-lasting influence of bFGF on neuronal development could explain the lack of effect on pontine cholinergic, the relatively minor effect on mesencephalic dopaminergic, and the pronounced early effect on septal cholinergic cells. However, the action of bFGF on dopaminergic cells of the mesencephalic cultures, in contrast to its effect on the cholinergic neurons of the septal cultures, depends on cell proliferation. It is likely, therefore, that the effect of bFGF on the dopaminergic cells is mediated by glia cells and that its only direct effect on neurons in our cultures is on the septal cholinergic cells. The stimulatory actions of insulin and the IGFs on neural development and differentiation are most likely very widespread and, at least *in vitro*, not limited to a specific time in development. This family of growth factors therefore seem unlikely candidates for target-derived, neuron-population-specific neurotrophic factors. Nevertheless, their time- and site-specific presence could be required during development and their function during this time might be different from later, more general, stimulating influences on many biochemical parameters.

Our finding that NGF and bFGF directly influence cholinergic neurons of the basal forebrain, but not the other studied neuronal populations, invites an interpretation that seems interesting in another context than discussed so far. It can be speculated that septal cholinergic neurons have a higher intrinsic plasticity than pontine cholinergic or mesencephalic dopaminergic neurons and therefore can respond with changes in morphology and biochemistry to different trophic or specifying factors (Varon and Adler, 1980) or other treatments. This possibility seems particularly interesting, since central cholinergic mechanisms have long been recognized to be instrumental in memory processes (see Squire and Davis, 1981; Singh et al., 1985).

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S-100β and Insulin-Like Growth Factor-II Differentially Regulate Growth of Developing Serotonin and Dopamine Neurons In Vitro

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T study the phenotypic specificity of S-100β and insulin-like growth factor II (IGF-II) for developing m noamine neurons, serotonin (5-HT) neurons from the embryonic day 14 (E14) rostral raphe or dopamine (TH) neurons from the substantia nigra/ventral tegmental area were cultured for 3 days in vitro (3 DIV) in the presence of these factors. Neuronotrophic effects were analyzed by computer-assisted morphometry of 5-HT and TH-immunoreactive neurons. S-100β and IGF-II differentially regulated the growth of 5-HT and TH neurons but did not affect their survival. S-100β significantly increased several parameters of neurite outgrowth by 5-HT neurons but inhibited the spatial extent (field area) of TH neurites. IGF-II promoted growth of cell bodies of both phenotypes, but only stimulated neurite outgrowth by TH neurons. S-100β and IGF-II differentially affected the number of GFAP immunoreactive cells fr m raphe and substantia nigra, but these effects did not correlate with the specificity of neuronotrophic effects. S-100β and IGF-II immunoreactivities were expressed in glial cultures derived from the same brain regions, raising the possibility that these factors have autocrine effects on glia as well as paracrine actions on neurons. The results of this study suggest that specificity of neuronotrophic factors for particular embryonic neurons may be correlated with their neurotransmitter phenotype.

Key words: 5-HT, monoamines, trophic factors, embryonic neurons, neurite outgrowth

INTRODUCTION

Previous studies suggest that neuronotrophic factors have characteristic effects on different neuronal phenotypes (Assouline et al., 1987; Azmitia et al., 1990a,b; Casper et al., 1991; Ferrari et al., 1989, 1991; Hofmann, 1988; Honegger and Lenoir, 1982; Knusel et al., 1990). To test the hypothesis that expression of neurotransmitter phenotype imparts specific growth response repetoires to developing neurons, two types of monoaminergic neuron were chosen which develop in close proximity in the rat embryo (König et al., 1987). Cultured embryonic serotonin (5-HT) and dopamine (TH) neurons were tested for their growth responses to two putative neuronotrophic factors, S-100β and insulin-like growth factor II (IGF-II).

S-100β is a calcium binding protein that is synthesized by glia and regulates the proliferation and cytoskeletal organization of these cells (Moore, 1965; Selinfreund et al., 1990). Interestingly, this protein is localized in midline glial structures of the brainstem in the rat embryo (Gomez et al., 1990; Landry et al., 1990; Van Hartesveldt et al., 1986), which are located adjacent to developing 5-HT neurons (Lauder and Wilkie, unpublished observations; Lidov and Molliver, 1982; Wallace and Lauder, 1983). Dopamine neurons also align along radial glia in the developing mesencephalon (Shults et al., 1990), which may also express S-100β. Neuronotrophic signalling between 5-HT neurons and postnatal astrocytes appears to involve regulation of glial S-100β by 5-HT_{1A} receptors (Azmitia et al., 1990a; Whitaker-Azmitia et al., 1990). S-100β promotes neurite outgrowth by cultured 5-HT and cortical neurons (Azmitia et al., 1990b; Kligman and Marshak, 1985; van Eldik et al., 1988; Winningham-Major et al., 1988, 1989), and some catecholamine neurons (Nakagawara et al., 1986).

Insulin-like growth factors are peptides related to the hormone insulin (Froescher et al., 1985; Gammeltoft, 1989; Humbel, 1984), that are found in highest concentrations in serum of fetal rats (Moses et al., 1980). IGF-II mRNA is expressed in adult rat brain, but highest levels are present prenatally (Brown et al., 1986; Hasselbacher et al., 1985; Rotwein et al., 1988; Soares et al., 1986). The location of this expression is uncertain since in situ hybridization studies indicate that only the choroid plexus

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expresses detectable levels of IGF-II mRNA in adult rat brain (Beck et al., 1987; Hynes et al., 1988; Stylianopoulou et al., 1988a,b). However, cultured postnatal astrocytes express mRNA for IGF-II and one of its binding proteins (IGFBP-2; Ballotti et al., 1987; Olson et al., 1991; Rotwein et al., 1988). Astrocyte-conditioned medium also contains these binding proteins (Han et al., 1988; Ocrant et al., 1990; Olsen et al., 1991). Receptors for IGF-II are found on both neurons and glia (Burgess et al., 1986; Han et al., 1987; Shemer et al., 1987). This growth factor promotes neurite outgrowth by cultured forebrain neurons (Svrzic et al., 1989), neuroblastoma (Mill et al., 1985) and sympathetic ganglia (Recio-Pinto and Ishii, 1984; Recio-Pinto et al., 1986). Preliminary studies indicate that IGF-II antibodies inhibit the growth of cultured embryonic dopamine, but not 5-HT neurons (Han, Liu and Lauder, unpublished observations).

The present study demonstrates phenotypic specificity of S-100β and IGF-II for cultured embryonic monoamine neurons, as well as specific effects on glia derived from the same brain regions.

MATERIALS AND METHODS

Dissociated Cell Cultures

To assess neuronotrophic effects of S-100β and IGF-II, cultures containing 5-HT neurons from the embryonic day 14 (E14) rostral raphe (RR) or tyrosine hydroxylase (TH) neurons from the dopaminergic substantia nigra/ventral tegmental area (SN) were prepared as previously described (König et al., 1987). Cells suspended in medium [Basal Medium Eagle's + 10% Nuserum (Collaborative Res. Incorp.) + pen/strep/dextrose] were plated onto glass coverslips coated with poly-D-lysine (10 μg/ml, Sigma) in 12-well tissue culture plates at an initial plating density of 1.0×10^6 cells/ml (1 ml/well). S-100β (Sigma Chem. Co.) or IGF-II were added to culture media at the time of plating. Cells were incubated at 37°C in an atmosphere of 5% CO_2, 95% air, and 100% humidity for 3 DIV prior to fixation and immunocytochemistry for 5-HT, TH or glial fibrillary acidic protein (GFAP).

For immunocytochemistry of S-100β or IGF-I/II, separate cultures were prepared from E14 RR or SN, as described previously (Wilkie and Lauder, 1988), and grown for 14 DIV. These cultures consist largely of glial elements, including radial-like glia and astrocytes, as well as a few other non-neuronal cells.

Immunocytochemistry

At 3 DIV, cultures were rinsed with Hanks' buffered salt solution (HBSS) and fixed with 4% paraformaldehyde in 70 mM phosphate buffer. Cultures were then rinsed in phosphate buffered saline (PBS), permeabilized with 0.2% Triton X-100, and immunostained using the avidin-biotin (ABC) peroxidase method (Vector Labs, Inc.) with specific rabbit polyclonal antisera against 5-HT-hemocyanin conjugates (Wallace et al., 1982), tyrosine hydroxylase (TH, Boehringer Mannheim Co.), glial fibrillary acidic protein (GFAP, Accurate Chem.), S-100β (Sigma Chem. Co.) or a monoclonal antibody raised against somatomedin-C (Russell et al., 1984). The somatomedin-C (IGF-I) monoclonal antibody (Gillespie et al., 1987) has 50% crossreactivity with IGF-II (Underwood, personal communication), thus the use of the term IGFI/II to designate immunoreactivity observed with this antibody. Specificity of the S-100β antiserum was established by blocking of immunoreactivity with purified S-100β (Sigma). Specificity of other antisera have been well established in previous studies.

Number of Neurons or Glia

For each experiment, the number of 5-HT or TH immunoreactive (IR) neurons or GFAP-IR glia were counted in 3 cultures/treatment group at 200x using an ocular grid. Fifty grid areas were counted per culture = a sampling area of 2.6 mm^2 (a total of 150 grid areas/experiment). Data were expressed as the mean number of cells/mm^2/culture. Data from three separate experiments were averaged and expressed as the mean number of cells/mm^2/treatment ± SEM (Table I). Statistical analysis was performed using Fisher's test following analysis of variance ($P<.05$; Dowdy and Wearden, 1983).

Morphometry

Cell size, shape, and complexity of neurite outgrowth were measured for 5-HT and TH-IR neurons as indices of neuronotrophic effects of S-100β and IGF-II. Morphometry was performed using a custom designed computer imaging system, which automatically measured soma area, field area, form factors, number of neurites, length and bending of initial, intermediate and terminal neurite segments and stored images for further analysis. Details of these parameters have been described previously (Lieth et al., 1990; Liu and Lauder, 1991). Neurites were traced at 200× magnification and cell bodies were measured at 400×. Neurites measured were assumed to consist mainly of dendrites, since thin caliber, long axon-like processes (most of which passed out of the field of view) were not measured. In each experiment, 20 randomly selected neurons were analyzed from 3 cultures per treatment group. Data from 3 experiments (total of 60 cells per group) were averaged to obtain means for each parameter ± SEM (Figs. 3, 4). Data were analyzed using Fisher's test following analysis of variance (Dowdy and Wearden, 1983; $P<.05$).

RESULTS

S-100β and IGF-II exhibited differential trophic activity for 5-HT or tyrosine hydroxylase (TH) neurons

TABLE I. Effects of S-100β and IGF-II on Number of Immunoreactive Neurons and Glia in Cultures From Day 14 Embryos†

Cells	Treatment	Mean	SEM
5-HT-RR	CONT	4.56	0.71
	S-100β (500)	3.41	0.65
	S-100β (50)	3.58	0.76
	S-100β (5)	3.80	0.65
	IGF-II (20)	3.50	0.76
	IGF-II (10)	4.23	0.54
GFAP-RR	CONT	90.07	4.24
	S-100β (500)	104.78*	2.07
	S-100β (50)	93.37	4.76
	S-100β (5)	97.70	4.25
	IGF-II (20)	99.27	3.82
	IGF-II (10)	107.40*	1.30
TH-SN	CONT	30.50	6.20
	S-100β (500)	23.23	2.61
	S-100β (50)	23.61	3.40
	S-100β (5)	30.70	6.60
	IGF-II (20)	34.20	6.70
	IGF-II (10)	33.50	4.00
GFAP-SN	CONT	10.63	1.67
	S-100β (500)	8.11*	0.45
	S-100β (50)	12.88	1.00
	S-100β (5)	9.91	1.06
	IGF-II (20)	11.79	0.71
	IGF-II (10)	12.40	0.61

†Abbreviations: 5-HT-RR: 5-HT neurons from the rostral rhombencephalon(B4-9); TH-SN: tyrosine hydroxylase (dopamine) neurons from the substantia nigra/ventral tegmental area of the mesencephalon (A9-10); GFAP: glial fibrillary acidic protein; CONT: control. Means are from counts of 150 grid areas (50/expt.) and represent average number of cells/mm^2 (SEM: standard error of the mean). Concentrations of S100-β and IGF-II in parenthesis (ng/ml).
*Significantly different from control ($P<.05$).

and associated glia when added to cell culture medium for 3 DIV. Stimulatory effects on neurite outgrowth demonstrated striking phenotypic specificity of S-100β for 5-HT neurons and IGF-II for TH neurons (Figs. 1–3), whereas cell soma growth of both phenotypes was increased by IGF-II. Glial cells present in these cultures also exhibited regional specificity in their responses to these factors (Table I), but this did not directly correlate with neuronotrophic effects. Immunoreactivity for S-100β and IGF-I/II was present in glial cultures from the embryonic day 14 (E14) rostral raphe (RR) and substantia nigra/ventral tegmental area (SN) at 14 days in vitro (14 DIV, Fig. 4).

Neuronal Cell Soma Growth Is Stimulated by IGF-II, but Not S-100β

The size of 5-HT cell somas (soma area; Fig. 2A) was significantly increased by IGF-II (20 ng/ml), compared to other conditions. The shape of these cell bodies was also altered, (i.e., soma form factor was increased, indicating cell bodies were more rounded) after treatment with 500 ng/ml S-100β compared to 10 ng/ml IGF. Both doses of IGF-II produced larger TH cell somas compared to 5 ng/ml S-100β, which decreased soma area compared to controls (Fig. 3A).

Neurites of 5-HT and TH Neurons Are Differentially Affected by S-100β and IGF-II

Spatial extent of neurites and shape of neuritic arbors. Neurites of 5-HT neurons treated with 500 ng/ml S-100β covered a larger field area (surface area; Fig. 2B) than controls or those exposed to 50 ng/ml S-100β. The highest dose of IGF-II decreased the field area form factor of 5-HT neurons, indicating that neuritic arbors were more bipolar in shape. Both doses of IGF-II increased the field area of TH neurites compared to S-100β, which had an inhibitory effect at the lowe. dose (Fig. 3B).

Number of terminal segments (neurite complexity). 5-HT neurons treated with S-100β (500 ng/ml) had more terminal neurite segments than controls or those treated with IGF-II at 10 ng/ml (Fig. 2C). The number of terminal segments on TH neurons was increased by 10 ng/ml IGF-II compared to all other treatments (Fig. 3C).

Number of neurites. The number of primary neurites on 5-HT neurons was increased by S-100β (500 ng/ml) compared to controls or 10 ng/ml IGF-II (Fig. 2D). TH neurons treated with IGF-II (10 ng/ml) had more primary neurites compared to all other treatments (Fig. 3D).

Initial segment length and bending. Variations in the length or bending of segments indicates that changes have occurred in the longitudinal or directional growth of neurites. TH neurons treated with S-100β (50 ng/ml; 5 ng/ml) or IGF-II (10 ng/ml) had significantly shorter initial segments compared to controls, whereas those exposed to 20 ng/ml IGF-II had longer initial segments compared to the lower dose of IGF. Values for initial segment bending of TH neurites were significantly increased (indicating segments were more curved) after treatment with 20 ng/ml IGF-II compared to S-100β (50 ng/ml; 5 ng/ml) or 10 ng/ml IGF-II. Initial segments of TH neurons treated with 500 ng/ml S-100β were more curved than those treated with 50 ng/ml or IGF at 10 ng/ml. S-100β at 50 ng/ml produced straighter initial segments compared to controls or the lowest dose of S-100β.

Terminal segment length and bending. The length of terminal segments of 5-HT neurons was increased by IGF-II at 10 ng/ml compared to S-100β at 50 ng/ml. Values for terminal segment bending of TH neurites was increased by S-100β at 500 ng/ml or IGF at 20 ng/ml compared to S-100β at 50 ng/ml or IGF at 10 ng/ml.

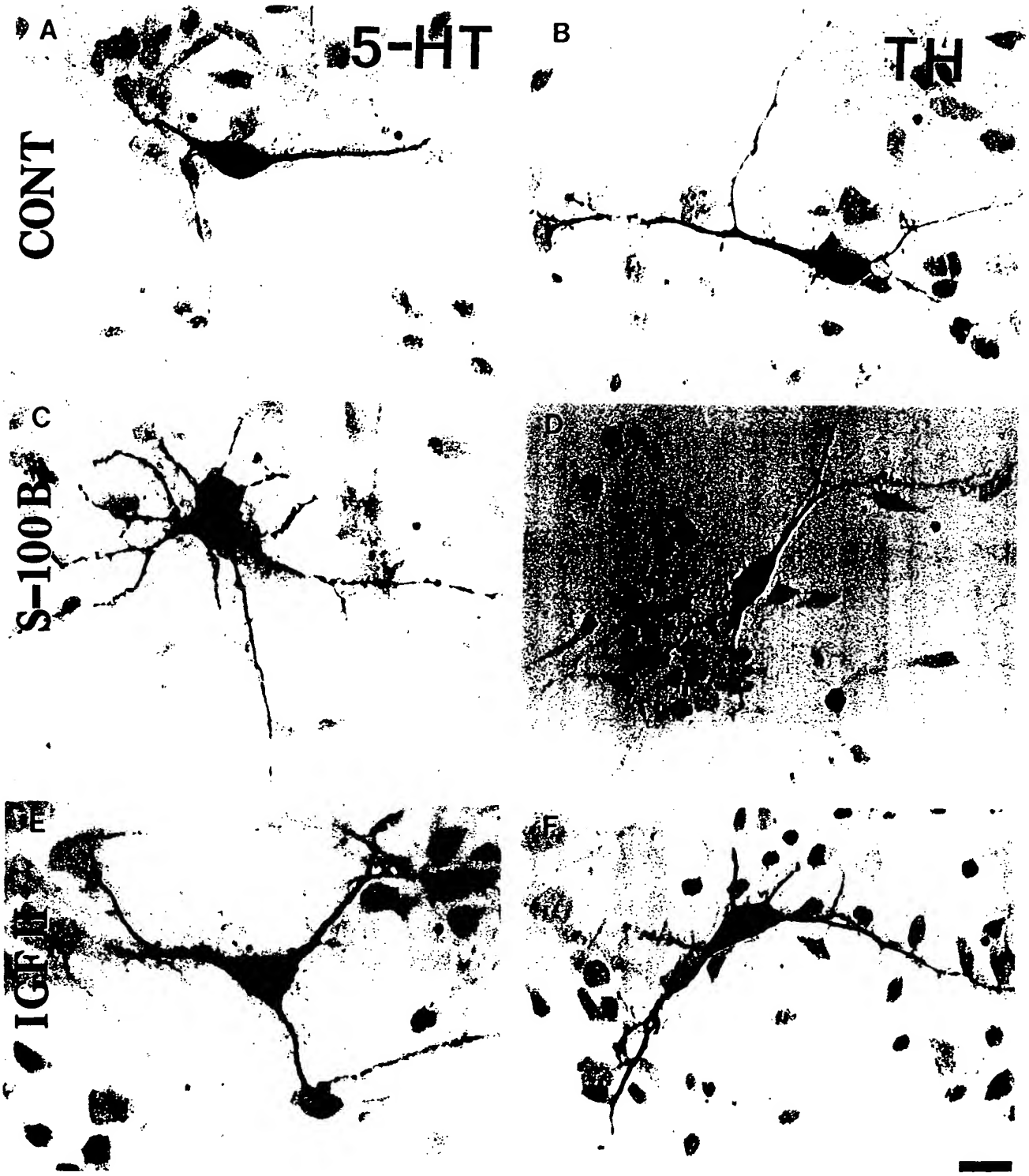

Fig. 1. Typical appearance of 5-HT **(A,C,E)** and TH **(B,D,F)** neurons treated with S-100β (500 ng/ml; C,D) or IGF-II (10 ng/ml; E,F). Note the increased complexity of cells C and F compared to controls (A,B). Bar = 12 μM.

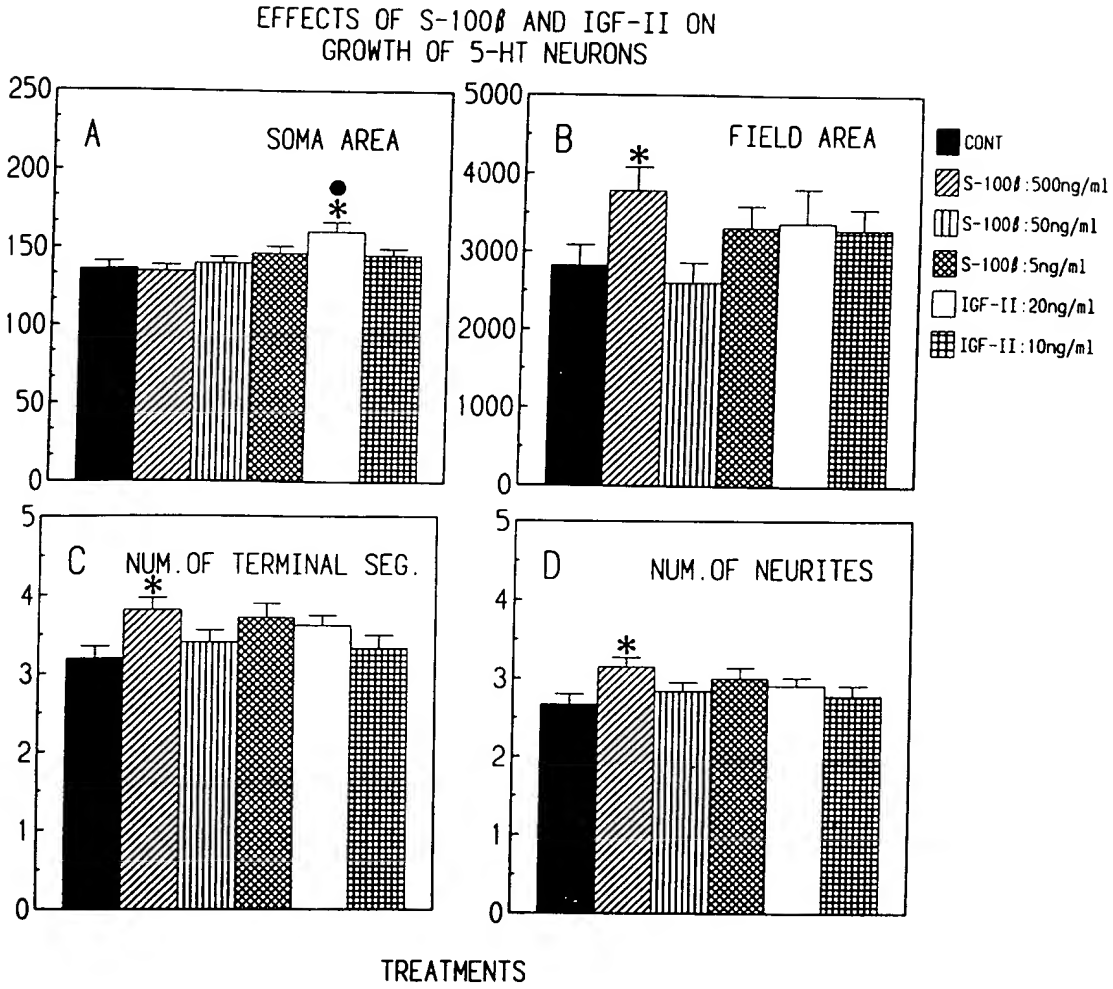

Fig. 2. Effects of treatments on growth of E14 5-HT neurons as determined by morphometric analysis of immunocytochemically stained cells. *Significantly different from control ($P<.05$). •Significantly different from IGF-II or S-100β ($P<.05$).

Glial Cells Show Regional Specificity in Their Responses to S-100β and IGF-I/II

S-100β (500 ng/ml) and IGF-II (10 ng/ml) increased the number of glial fibrillary acidic protein immunoreactive (GFAP-IR) cells in rostral raphe cultures (Table I). In contrast, the same concentration of S-100β reduced the number of GFAP-IR cells in substantia nigra cultures, whereas IGF-II had no significant effect. Neither factor affected neuronal survival.

DISCUSSION

5-HT and TH Neurons Exhibit Differential Responses to Neuronotrophic Factors

Embryonic 5-HT and TH neurons responded selectively to S-100β or IGF-II. Neurite outgrowth by 5-HT neurons was enhanced by S-100β, wherea some inhibitory effects on TH neurons were found (decreased soma and field areas). Growth of TH cell bodies and neurites was promoted by IGF-II, which had no significant effects on neurite outgrowth by 5-HT neurons, although cell body size was enhanced. These results are consistent with previous studies indicating differential effects of these growth factors on 5-HT and dopamine neurons (Azmitia et al., 1990a; Bradshaw et al., 1991; Casper et al., 1991; Knusel et al., 1990), and support the hypothesis that expression of neurotransmitter phenotype imparts specific growth response repertoires to developing neurons.

Possible Mechanisms Underlying Neuronotrophic Effects

The specific responses of cultured 5-HT and TH neurons to S-100β and IGF-II suggest that these cells may be heterogeneous with respect to expression of growth factor receptors. Previous studies have demonstrated that a short exposure to S-100β is sufficient to promote neurite

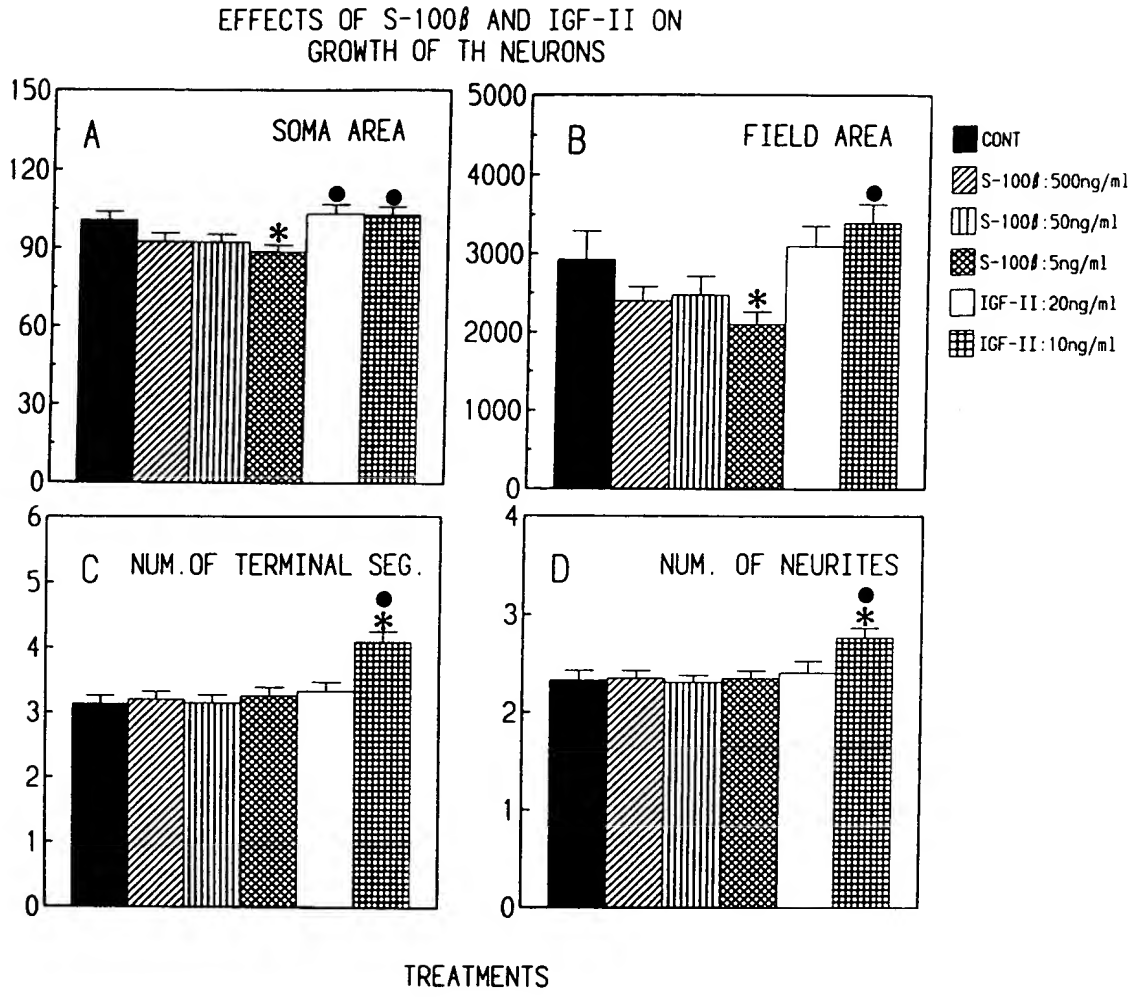

Fig. 3. Effects of treatments on growth of E14 TH neurons as determined by morphometric analysis of immunocytochemically stained cells. Significance as in Figure 2.

outgrowth, suggesting that this effect may occur by receptor-mediated mechanisms (Marshak, 1990; Winningham-Major et al., 1988). S-100β has also been reported to regulate microtubule assembly and stability in rat brain extracts (Hesketh and Baudier, 1986) and may act as a neuromodulator in nervous tissue (Baudier et al., 1985; Donato, 1984; Endo and Hidaka, 1983; Gomez et al., 1990; Patel et al., 1983). Although no data are available regarding the presence of S-100β receptors on embryonic neurons, there are reports of S-100β binding sites on various types of neuronal membranes (Donato, 1977; Donato et al., 1975). Since 5-HT neurons respond more strongly to S-100β than do TH neurons, it is possible that they express more abundant S-100β receptors, or that these receptors have a higher binding affinity.

IGFs interact with two types of receptor on neurons and glia to exert their effects on growth and differentiation (see Czech, 1989). The type I IGF receptor binds IGF-I and IGF-II with high affinity and has tryosine kinase activity, whereas the type II IGF receptor binds IGF-II with considerably higher affinity than IGF-I (Gammeltoft, 1989; Rechler and Nissley, 1985; Roth, 1988). Neurite outgrowth is stimulated by IGF-II via activation of the type I IGF receptor (Gammeltoft et al., 1985; Kasuga et al., 1982; Nielsen et al., 1991), whereas the type II IGF receptor appears to be involved in the internalization and degradation of IGF-II (Nielsen et al., 1991). Given the greater responsiveness of TH neurons to IGF-II, it is possible that these cells express type I IGF receptors.

Lack of Correlation of Neuronotrophic Activity With Effects of Factors on Glia

S-100β and IGF-II altered the number of glia cells in cultures containing 5-HT or TH neurons. This could potentially have contributed to neuronotrophic effects. However, the responses of 5-HT and TH neurons did not

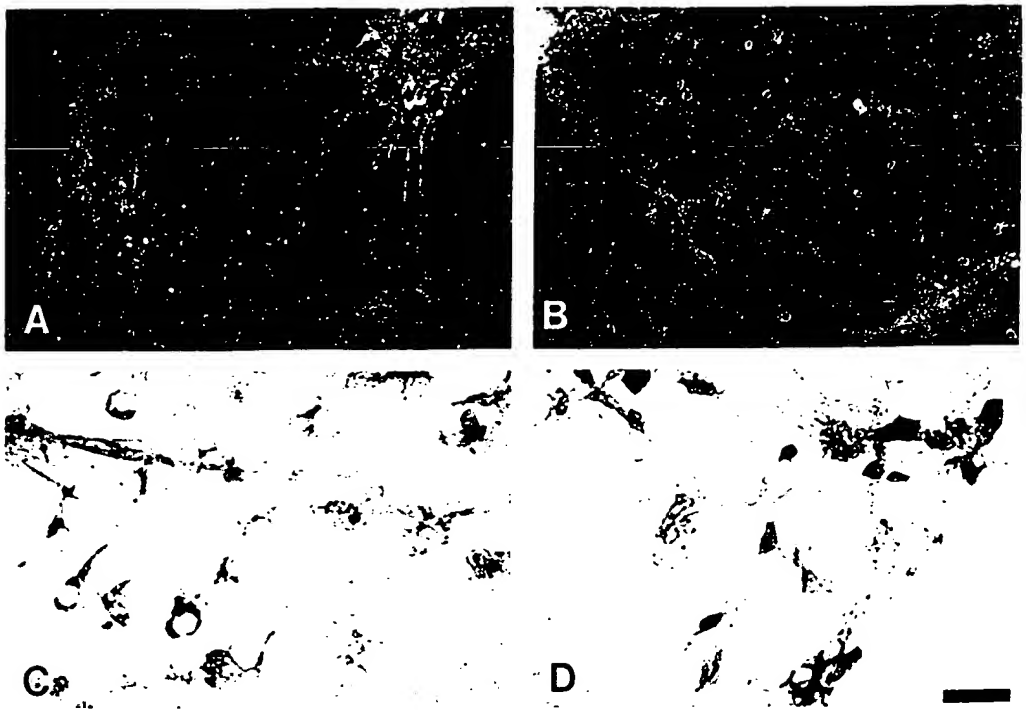

Fig. 4. Expression of immunoreactivity for S-100β (**A,B** = darkfield) and IGF-I/II (**C,D** = brightfield) in glial cultures from embryonic E14 rostral raphe (A,C) and substantia nigra (B,D) prepared according to the method of Wilkie and Lauder (1988), and grown for 14 days in vitro (DIV). Bar = 44 μm (A,B) or 17.6 μm (C,D).

correlate with the regional specificity of glial effects. Both S-100β (500 ng/ml) and IGF-II (10 ng/ml) increased the number of glia in raphe cultures, but only S-100β affected neurite outgrowth. Moreover, cell soma size (soma area) was stimulated by a different dose of IGF-II. Likewise, IGF-II did not alter the number of glia in substantia nigra cultures, whereas it had dose-dependent effects on the growth of TH cell bodies and neurites. Moreover, S-100β (500 ng/ml) decreased the number of glia in nigral cultures, but did not affect growth of TH neurons. Therefore it does not appear that changes in the number of glia can explain the effects of these factors on neurons in the same cultures.

The specific responses of glia to S-100β and IGF-II are particularly interesting in light of the expression of these factors by glial cultures derived from the same embryonic brain regions (Fig. 4). These results provide evidence for regional heterogeneity of glial responses to trophic factors during embryogenesis, and suggest that S-100β and IGF-II may have autocrine actions on glia as well as paracrine effects on neurons.

CONCLUSIONS

Both monoamine neurons and associated glia from the embryonic raphe and substantia nigra responded in a regionally specific manner to S-100β and IGF-II in vitro. This may reflect the differential expression of receptors or other signal transduction mechanisms by these cells. Glial cultures derived from the same brain regions expressed immunoreactivity for S-100β and IGF-I/II suggesting that these factors may be produced by non-neuronal cells in the embryo. The striking phenotypic specificity of S-100β and IGF-II for embryonic 5-HT or TH neurons suggests that these neuronotrophic factors may play important roles in the construction of developing monoaminergic circuitry.

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ng/ml), a clear enhancement of all bands was seen, with least effect on BP-5. Although tIGF-I (150 ng/ml) combined with FGF (25 ng/ml) showed 5-fold more biological activity as amino acid incorporation (data not shown), it was less effective than the IGF-I/FGF combination in enhancing BP-3, similarly or sometimes less effective on BP-2 and BP-5, and similarly effective on BP-4.

Northern Analysis

Using specific cDNA probes for BP-2, BP-4, and BP-5, we identified m-RNA for all BPs in total RNA extracted from fresh 1PN, 6PN OB and cultured OB. BP-3 mRNA was not detected. IGF-I and FGF both increased expression of BP-2 and BP-4, while BP-5 mRNA was increased by IGF-I and reduced by FGF.

CONCLUSION

In newborn rat OB organ culture, IGF-I and FGF in combination support the survival and differentiation of specialized cells. IGFBPs -2, -3, -4, and -5 are expressed and their synthesis and turnover specifically modulated by these growth factors.

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IGF-I Supports the Survival and/or Differentiation of Multiple Types of Central Nervous System Neurons

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Insulin-like growth factors (IGFs) have been identified in various animal species as polypeptides that act to stimulate growth and/or differentiation in a variety of tissues.[1] IGF-I is expressed in the central nervous system (CNS) in a developmentally and regionally specific fashion[2] in both neuronal and glial cells,[3] and a truncated form, (des 1-3) IGF-I, appears to be the main species in brain.[4] In both rat and human brain, IGF-I expression is high during development, compared to low levels observed in the adult. The biological effects of IGF-I and IGF-II appear to be mediated by the IGF-I receptor, which is found in a variety of tissues, including a fairly ubiquitous distribution in the nervous system.[5,6]

The physiological functions of the IGFs in the developing and mature nervous system are not yet clearly delineated, but results from a number of studies strongly suggest a role for IGFs in the regulation of neuronal development and maintenance in both the central and peripheral nervous systems.[7–14] In order to identify additional cellular targets of IGF-I in the CNS and to more fully characterize its potential survival and differentiation activities, we have examined IGF-I effects on a variety of neurons derived from the CNS.

IGF-I PROMOTES SURVIVAL OF DEVELOPING CNS NEURONS

Primary cultures of postmitotic neurons derived from various regions of the embryonic rat brain were plated at low density in serum- and insulin-free N2 medium[15] containing 0.05% BSA. Under these conditions, survival and neurite outgrowth were limited and decreased with time in culture. Neuronal survival was assessed by a fluorimetric viability assay in which a fluorescein diacetate analogue, calcein AM, was taken up and cleaved by viable cells to a fluorescent product that was measured in a plate-reading microfluorimeter. Addition of IGF-I to cultures resulted in a dose-dependent enhancement of neuronal survival in cultures derived from embryonic age (E)14 mesencephalon, E 14.5 spinal cord, E 18 cerebral cortex, postnatal day 10 retina, and E 16 basal forebrain with EC_{50} values ranging between 0.05 and 0.1 nM. Although not quantified, extensive neurite outgrowth and branching were also observed in IGF-I-treated cultures. Maximal neuronal survival was typically 1.5–2.5 over basal (TABLE 1).

EFFECTS OF IGF-I ON CHOLINERGIC AND DOPAMINERGIC NEURONS

It was previously reported that IGF-I and -II enhanced specific dopamine uptake in mesencephalic cultures.[14] IGF-I could act to increase dopamine uptake in existing dopaminergic neurons, increase the survival and/or the rate of dopaminergic differentiation, or affect phenotypic switching in these cultures. We therefore evaluated the number of tyrosine hydroxylase immunoreactive neurons in cultures of ventral mesencephalic neurons after 7 days in the absence or presence of 50 nM IGF-I. IGF-I resulted in a 2-fold increase in the number of neurons stained with an antibody to tyrosine hydroxylase. Tyrosine hydroxylase enzyme activity was also found to increase approximately 2-fold in identically treated cultures. To determine IGF-I effects on cholinergic neurons, cultures were established from embryonic rat spinal cord or basal forebrains, and maintained in serum- and insulin-free N2 medium in the presence or absence of IGF-I. Choline acetyltransferase (ChAT) activity was assessed after 2 to 5 days in culture. IGF-I resulted in a dose-dependent increase in spinal cord ChAT activity (up to 4-fold over uninduced controls) with an $EC_{50} \cong 0.5$ nM. In untreated control cultures, ChAT activity progressively declined, probably due to cholinergic neuron death or dedifferentiation. Addition of 10 nM IGF-I at

TABLE 1[a]

Neuron Type	Fold Increase over Control Survival ± Std. in the Presence of 10 nM IGF-1	
Cortical	1.92	0.17
Basal forebrain	1.94	0.21
Mesencephalic	1.77	0.28
Retinal	2.37	0.71
Spinal cord	1.8	0.09

[a]Cells were seeded at 6×10^4 cells/cm^2 in serum- and insulin-free N2 medium on poly-*l*-ornithine and laminin substrates and assayed after 48 h as described in the text.

culture initiation resulted in only a 16% loss of the ChAT activity measured at culture initiation compared to a 75% loss in untreated control cultures. IGF-I also promoted approximate 2-fold increases in ChAT activity and in the number of acetylcholinesterase-positive neurons in basal forebrain cultures, with a dose-response relationship similar to that observed in spinal cord cultures. In summary, these results suggest that IGF-I promotes the rate of differentiation and/or the survival of cholinergic and dopaminergic neurons. IGF-I might also act on these neuronal populations to recruit additional neurons to a cholinergic or dopaminergic phenotype via phenotypic switching.

The multiple neurotrophic activities of IGF-I contrast sharply with more restricted activities or target populations of other factors (e.g., nerve growth factor). It is likely that the wide distribution of IGF-I receptors in the brain[5,6] compared with the highly regional appearance of receptors for other neurotrophic factors, accounts for the breadth of IGF-I activity in the CNS, and suggests that the IGFs may have a fundamental role in neuronal development, maintenance, and recovery from injury.

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IGF-I and IGF-II Protect Cultured Hippocampal and Septal Neurons against Calcium-mediated Hypoglycemic Damage

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Insulin and insulin-like growth factors I and II (IGF-I and IGF-II) have recently been shown to have biological activity in central neurons, but their normal functions and mechanisms of action in the brain are unknown. Since central neurons ar particularly vulnerable to hypoglycemia that results from isch mia or other insults, we tested the hypothesis that growth factors can protect central neurons against hypoglycemic damage *in vitro*. IGF-I and IGF-II (3–100 ng/ml) each pr vent d glucose deprivation–induced neuronal damage in a dose-dependent manner in rat hippocampal and septa! cell cultures. High concentrations of insulin (greater than 1 μg/ml) also protected neurons against hypoglycemic damage. Epidermal growth factor did not protect against hypoglycemic damage. Both IGFs and insulin were effective when administered 24 hr before or immediately following the onset of glucose deprivation. Direct measurements of intraneur r nal calcium levels and manipulations of calcium influx demonstrated that calcium influx and sustained elevations in intraneuronal calcium levels mediated the hypoglycemic damage. IGF-I and IGF-II each prevented the hypoglycemia-induced elevations of intraneuronal free calcium. Studies with xcitatory amino acid receptor antagonists and calcium chann l blockers indicated that NMDA receptors did, and L-typ calcium channels did not, play a major role in hypoglycemic damage. Taken together, these findings indicate that IGFs can stabilize neuronal calcium homeostasis and th reby protect against hypoglycemic damage.

Insulin and insulin-like growth factors I and II (IGF-I and IGF-II) are closely related polypeptides that are similar in their structures and are believed to have overlapping biological functions (Rinderknecht and Humbel, 1976, 1978a,b; Humbel, 1984; Czech, 1985; Froesch et al., 1985; Rechler and Nissley, 1985; Baskin, 1987; Baskin et al., 1988; Garofalo and Rosen, 1989; Knusel et al., 1990; Sara and Hall, 1990). Insulin regulates the uptake, cellular transport, and intermediary metabolism of small nutrient molecules such as glucose, amino acids, and fatty acids in muscle and adipose tissues. Recently, insulin and IGFs (and/or their mRNAs) have been localized in the CNS (Havrankova et al., 1978; Sara et al., 1982, 1986; Rechler and Nissley, 1985; Baskin et al., 1988; Rotwein et al., 1988), suggesting that they may have biological activity in the brain. In brain cell cultures, insulin, IGF-I, and IGF-II promote neuronal survival, neurite outgrowth, and gene expression (Bhat, 1983; Lenoir and Honegger, 1983; Mill et al., 1985; Recio-Pinto et al., 1986; Aizenman and DeVellis, 1987; Kyriakis et al., 1987; Avola et al., 1988; Knusel et al., 1990; Drago et al., 1991). Insulin, IGF-I, and IGF-II are each associated with a distinct cell surface receptor (Rechler and Nissley, 1985). The IGF-I receptor is structurally and functionally similar to the insulin receptor in that it possesses an α-subunit that binds the hormone-like agent and a β-subunit that has a tyrosine-specific protein kinase (Ebina et al., 1985; Ullrich et al., 1985, 1986). The IGF-II receptor is different in structure from the IGF-I receptor or insulin receptor and appears to be identical to an intracellular mannose-6-phosphate receptor (Morgan et al., 1986, 1987).

Insulin, IGF-I, and IGF-II can be produced in cultured neurons and have been reported to occur in the brain (Binoux et al., 1981; Weyhenmeyer and Fellows, 1983; Davies et al., 1986; Ballotti et al., 1987). mRNAs for both IGFs and insulin have been detected in many brain regions (Haselbacher et al., 1985; Young, 1986; Baskin et al., 1988; Rotwein et al., 1988). There is abundant evidence for the existence of receptors for insulin and IGFs in the brain (Raizada et al., 1982, 1988; Hill et al., 1986; Burgess et al., 1987; Mendlesohn, 1987; Waldbillig and LeRoith, 1987; Bohanon et al., 1988; Lesniak et al., 1988; Garofalo and Rosen, 1989). IGF-I receptor mRNA is widely distributed in the brain, whereas insulin receptor mRNA is restricted to certain areas and appears to be coexpressed with the IGF-I receptor mRNA (Baron-Van Evercooren et al., 1991). Receptors for IGF-I and IGF-II have been found to be located in hippocampus (Lesniak et al., 1988; Araujo et al., 1989). IGF-II receptor mRNA distribution in brain has not been examined.

A continuous supply of glucose is necessary for the normal functioning and survival of mammalian central neurons. Hypoglycemia results in increased utilization of endogenous substrates, depletion of ATP, membrane depolarization, extracellular accumulation of excitatory amino acids, loss of neuronal ion homeostasis, and ultimately neuronal death (Siesjo et al., 1988). Calcium normally serves physiologically important functions as a second messenger regulating neuronal plasticity (Lynch et al., 1983; Kater et al., 1988). However, excessive and sustained elevations in intracellular calcium are involved in neuronal degeneration caused by metabolic and environmental insults (Choi, 1988; Siesjo et al., 1988; Mattson, 1992). In the CNS, the excitatory neurotransmitter glutamate contributes to neuronal vulnerability to insults such as hypoglycemia and hyp-

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oxia by enhancing calcium influx (Choi, 1988). The cellular and molecular mechanisms that normally protect neurons against such insults are largely unknown. In the present study we employed hippocampal and septal cell cultures to test the hypothesis that IGFs can stabilize neuronal calcium homeostasis and protect central neurons against hypoglycemic injury.

Materials and Methods

Hippocampal and septal cultures. The hippocampal and septal culture methods employed were similar to those of Mattson and Kater (1988) and of Hartikka and Hefti (1988), respectively. Briefly, rat hippocampi and septal areas were obtained from 18 d Sprague–Dawley fetuses and incubated for 15 min in a solution of 2 mg/ml trypsin in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution buffered with 10 mM HEPES (HBSS; GIBCO). The hippocampi and septal areas were then rinsed once in HBSS, followed by a 5 min incubation in HBSS containing 1 mg/ml trypsin inhibitor (Sigma), and a final rinse in HBSS. Tissues were then dissociated by trituration through the narrowed bore of a fire-polished Pasteur pipette and were distributed to polylysine-coated plastic culture dishes (Corning) containing 2 ml of Eagle's minimum essential medium (GIBCO) buffered with 10 mM sodium bicarbonate and supplemented 10% (v/v) with fetal bovine serum (Sigma), 2 mM L-glutamine, 20 mM KCl, 1 mM pyruvate, and 40 mM glucose. The culture density was 80–120 cells/mm² of culture surface. Cultures were maintained at 37°C in a 6% CO_2/94% room air, humidified incubator. All experiments were done with neurons that had been in culture for 8–15 d. In some experiments, glial proliferation was halted by exposing cultures to either 10 μM cytosine arabinoside (Ara-C) or 20 μM 5-fluoro-2'-deoxyuridine for 2–3 d (culture days 3–5).

Assessment of neuronal survival. Neuronal damage was assessed by our well-established morphological criteria, which correlate well with vital dye staining methods (Mattson et al., 1988). Briefly, cultures were visualized and photographed with a phase-contrast Nikon Diaphot inverted microscope. Neurons were scored as viable if they had neurites that were uniform in diameter and smooth in appearance, and somata that were smooth and round to oval in shape. In degenerating nonviable neurons, neurites were fragmented and beaded, and the soma was rough, swollen, vacuolated, and irregular in shape. Subsequent to these morphological changes, the degenerated neurons detached from the culture substrate. Viable neurons in premarked culture regions (four regions of approximately 1 mm²/culture) were counted immediately prior to and 18–24 hr following glucose deprivation. Statistical comparisons were done using pairwise Student's *t* tests.

Glucose deprivation and experimental treatments. Glucose deprivation was carried out by removing the culture maintenance medium and washing three times with glucose-free Locke's solution. Locke's solution contained (in mM) NaCl, 154; KCl, 5.6; $CaCl_2$, 2.3; $MgCl_2$, 1.0; $NaHCO_3$, 3.6; and HEPES buffer, 5. Calcium-deficient medium consisted of glucose-free Locke's solution lacking added calcium. Cultures were washed thoroughly (six 2 ml washes) with calcium-deficient Locke's immediately prior to glucose deprivation. Growth factors were prepared as 100–1000× stocks in water and were added directly to the cultures. Cultures were pretreated with growth factor for 24 hr prior to the onset of hypoglycemia, and the growth factors were included in the glucose-free medium during the period of hypoglycemia. Insulin (bovine) and calcitonin gene–related peptide (CGRP) were from Sigma, while IGF-I and IGF-II (human, recombinant) and epidermal growth factor (EGF; from mouse submaxillary glands) were from Boehringer Mannheim. D-γ-Glutamylglycine (DGG), DL-2-amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were from Tocris Neuramin; these agents were prepared as stocks in Locke's solution. Nifedipine, nimodipine, verapamil, diltiazem, flunarizine, lidoflazine, and trifluoperazine (Sigma) were prepared as 20–500× stocks in dimethyl sulfoxide. Equivalent volumes of vehicle were added to control cultures and did not affect neuronal survival.

Fura-2 measurements of intraneuronal calcium levels. For these studies cells were grown in glass-bottom dishes (Mat Tek, Inc.) coated with 0.05% polyethylenimine. Intraneuronal free calcium levels were measured 14–16 hr after the onset of glucose deprivation. The procedures for fura-2 fluorescence ratio imaging were similar to those of our past work (Mattson et al., 1989). Briefly, the cells were loaded at 37°C for 40 min with 4 μM fura-2 acetoxymethyl ester (Molecular Probes). The loaded cells were then washed two times with Locke's solution containing 2.3 mM $CaCl_2$ (with or without glucose) and incubated an additional 60 min prior to imaging to allow deesterification of the fura-2. Cells were viewed on an inverted Nikon microscope with a fluoro 40×, NA 1.3 objective and an intensified CCD Camera (Quantex). A Quantex imaging system with QFM software was used to acquire and process the images. Intracellular free Ca^{2+} levels were determined from the ratio of the fluorescence emission using two different excitation wavelengths (350 nm and 380 nm). Background fluorescence at each wavelength (background images were taken from regions of culture dish not containing cells) was subtracted from the cell image at that wavelength. The system was calibrated according to the procedures described by Grynkiewicz et al. (1985). Measurements were taken in neuronal cell bodies, and values represent the average free calcium level therein. Statistical comparisons were made using Student's *t* test.

Results

IGF-I, IGF-II, and insulin protect hippocampal and septal neurons against hypoglycemic damage

Incubation of rat hippocampal and septal cultures in glucose-free culture medium resulted in highly significant neuronal damage and death during 18 hr (hippocampal) or 24 hr (septal) exposure periods when compared with cultures maintained in medium containing 5–40 mM glucose (Fig. 1, Table 1). Approximately 85–95% of rat hippocampal and septal neurons degenerated during these glucose deprivation periods (n = 4–10 separate experiments for each treatment condition, 3–4 cultures/experiment). When rat hippocampal and septal cultures were pretreated for 24 hr with 100 ng/ml of either IGF-I or IGF-II and then deprived of glucose for 18–24 hr, there was a dramatic reduction in neuronal damage compared to glucose-deprived cultures not receiving a growth factor (Fig. 1, Table 1). Neuronal survival was increased to approximately 70% in IGF-treated cultures as compared with 5–15% in the untreated glucose-deprived cultures (n = 4–10 separate cultures/treatment group; $p < 0.001$). Both IGF-I and IGF-II were able to sustain hippocampal and septal neurons for up to 40 hr in the absence of glucose (n = 4 separate cultures). Insulin at 100 ng/ml did not protect neurons against hypoglycemic damage (Table 1). The data presented in the dose–response curve in Figure 2 demonstrate that very low concentrations of IGF-I and IGF-II (1–10 ng/ml, approximately 150–1500 pM) can significantly protect against neuronal damage in both hippocampal and septal cultures. Insulin at concentrations up to 300 ng/ml (approximately 60 nM) did not protect against hypoglycemic damage. However, higher concentrations of insulin (1 μg/ml or greater) did significantly protect both hippocampal and septal neurons against hypoglycemic damage (Figs. 1, 2). EGF from 1 to 100 ng/ml failed to prevent neuronal damage after glucose deprivation. Similarly, no effects were observed with 100 nM CGRP.

Since both rat hippocampal and septal cultures contained glia (predominately type I astrocytes; cf. Mattson et al., 1988), we determined whether reducing the number of glial cells in the culture would influence the protective effects of IGFs against glucose deprivation. Glial cell proliferation was inhibited by the addition of 10 μM Ara-C to the cultures (Fig. 3). Our preliminary experiments showed that adding Ara-C to hippocampal cultures reduced the number of astrocytes to less than 5–10% of the total cell number without significantly affecting the number of neurons. IGF-I and IGF-II were found to protect against neuronal death induced by glucose deprivation in glia-depleted cultures (Fig. 3). Similar results were obtained in septal cultures (data not shown). These data suggested that the neuroprotective actions of IGF-I and IGF-II were not mediated by glia, although

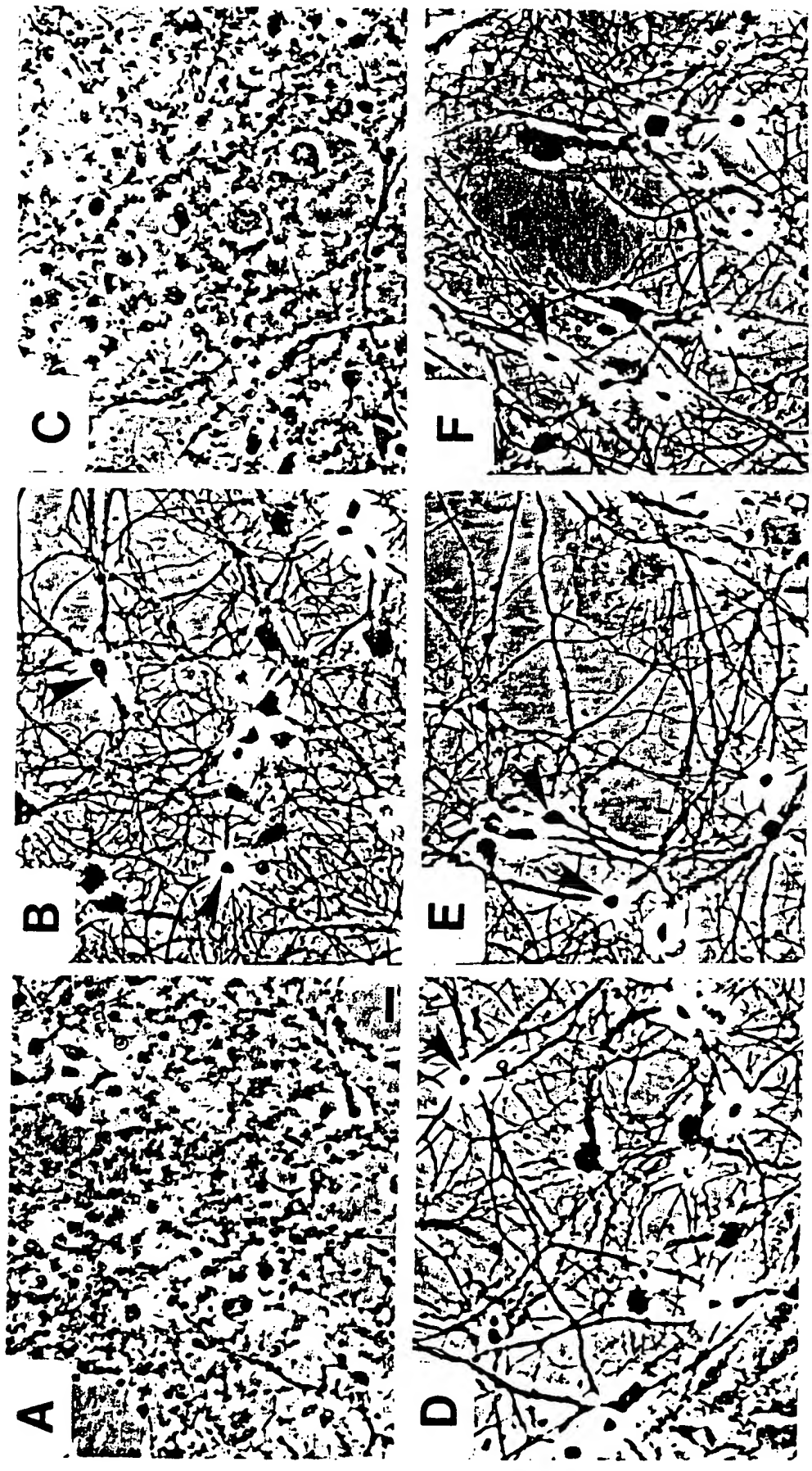


Figure 1. Hypoglycemic neuronal damage can be prevented by IGF-I, IGF-II, and insulin: phase-contrast micrographs from cell cultures of rat hippocampal neurons. *A*, After 18 hr of incubation in glucose-free medium; note massive damage to somata and neurites. *B*, After 18 hr of incubation in medium containing 20 mM glucose, somata (*arrowheads*) and neurites appear undamaged. *C*, A culture that had been pretreated with 100 ng/ml EGF for 24 hr prior to the onset of glucose deprivation is shown after 18 hr of glucose deprivation. *D*, A culture that had been pretreated with 1 μg/ml insulin for 24 hr prior to the onset of glucose deprivation is shown after 18 hr of glucose deprivation. *E*, A culture that had been pretreated with 100 ng/ml IGF-I for 24 hr prior to the onset of glucose deprivation is shown after 18 hr of glucose deprivation. *F*, A culture that had been pretreated with 100 ng/ml IGF-II for 24 hr prior to the onset of glucose deprivation is shown after 18 hr of glucose deprivation. Scale bar, 10 μm.

Table 1. Effects of growth factors and calcium removal on neuronal damage induced by glucose deprivation in cultured rat hippocampal and septal neurons

	Neuronal survival (% of initial number)	
	Hippocampus	Septum
0 glucose	6.2 ± 2.6	9.84 ± 2.5
5 mM glucose	74.82 ± 9.3*	80.82 ± 6.7*
20 mM glucose	77.91 ± 8.4*	79.38 ± 7.2*
40 mM glucose	83.75 ± 9.4*	87.56 ± 10.2*
0 glucose + 100 ng/ml IGF-I	63.49 ± 7.7*	81.22 ± 9.8*
0 glucose + 100 ng/ml IGF-II	59.89 ± 6.3*	74.89 ± 6.7*
0 glucose + 100 ng/ml insulin	13.38 ± 4.5	12.31 ± 4.4
0 glucose + 100 ng/ml EGF	14.29 ± 4.8	11.37 ± 3.8
0 glucose + 0 $[Ca^{2+}]_o$	75.62 ± 7.3*	81.48 ± 5.9*
0 glucose + 100 nM CGRP	3.50 ± 1.1	4.92 ± 2.4

Cultures were incubated in the presence of growth factors for 24 hr prior to exposure to glucose-free medium. Neuronal survival was assessed 18 hr (hippocampus) or 24 hr (septum) following the onset of glucose deprivation. Values represent the mean ± SEM of determinations made in 4–10 separate cultures per treatment group.

* $p < 0.001$, as compared to corresponding values for treatments with 0 glucose, 0 glucose + insulin, 0 glucose + EGF, or 0 glucose + CGRP.

the possibility that the few remaining glia played a role in the action of the IGFs cannot be ruled out.

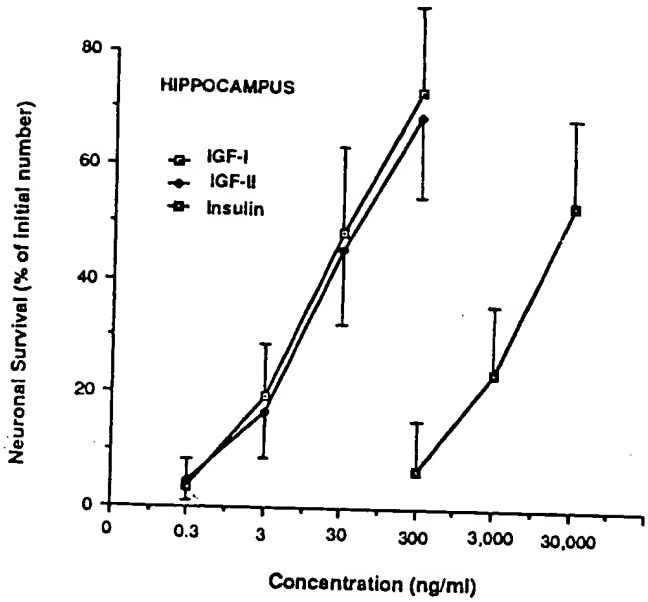
IGFs protect neurons against hypoglycemic damage by preventing a loss of cellular calcium homeostasis

Neuronal damage that occurs as the result of ischemia and excitotoxic insults results largely from aberrant elevations in intracellular calcium levels (Choi, 1988; Siesjo et al., 1988; Mattson, 1992). We therefore determined whether hypoglycemic neuronal damage was calcium dependent, and whether IGFs and insulin modified hypoglycemia-induced calcium responses. Hippocampal and septal cultures were incubated in medium lacking extracellular calcium during the period of glucose deprivation in order to prevent calcium influx through the plasma membrane (cf. Mattson et al., 1988). Hypoglycemic damage was significantly reduced in the cultures maintained in the calcium-deficient medium as compared to cultures maintained in the normal medium that contained 2.3 mM Ca^{2+} (Fig. 3, Table 1). These data indicated that calcium influx was necessary for hypoglycemic damage and suggested that glucose deprivation might result in a loss of neuronal calcium homeostasis. We therefore employed the calcium indicator dye fura-2 to determine the effects of hypoglycemia and growth factors on intraneuronal calcium levels.

Glucose deprivation caused a highly significant three- to five-fold elevation in intraneuronal calcium levels in both hippocampal and septal neurons during 14–16 hr periods (Table 2). IGF-I and IGF-II (100 ng/ml) each prevented the glucose deprivation–induced intraneuronal calcium increase. EGF did not prevent increase in intraneuronal calcium after glucose deprivation (Table 2). The results demonstrated that a loss of neuronal calcium homeostasis accompanied the hypoglycemic damage, and that IGFs stabilized intracellular calcium levels.

Involvement of excitatory amino acid receptors in hypoglycemic damage

Ischemic neuronal damage *in vivo* (Simon et al., 1984) and *in vitro* (Goldberg et al., 1987) involves excitatory amino acid



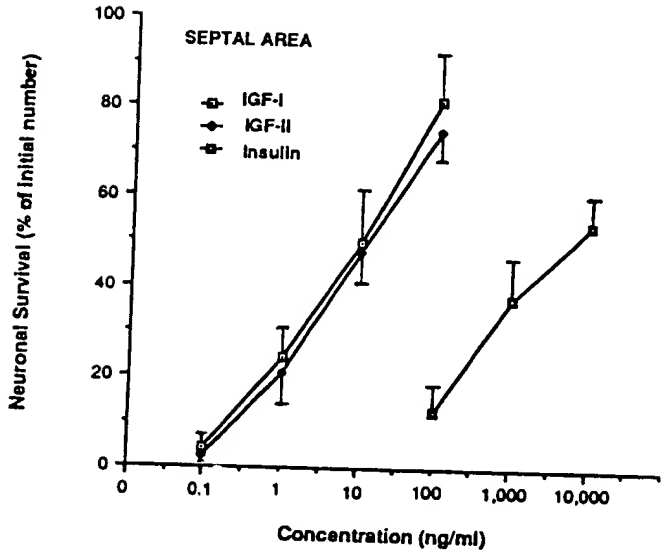


Figure 2. Dose–response curves for the protective effects of growth factors on rat hippocampal (*upper*) and septal (*lower*) cultures deprived of glucose. Cultures that had been pretreated for 24 hr with different concentrations of growth factors were exposed to glucose-free medium, and neuronal survival was assessed 18 hr (hippocampal cultures) or 24 hr (septal cultures) later. Values represent the mean and SEM of determinations made in three or four separate experiments.

receptor activation resulting in calcium influx. Since calcium influx was involved in the hypoglycemic damage in the present study, we assessed the involvement of excitatory amino acid receptors in the degenerative process. The NMDA receptor antagonist APV (100 μM) and the broad-spectrum glutamate antagonist DGG (100 μM) each prevented the elevation in intraneuronal calcium levels and neuronal damage induced by glucose deprivation (Fig. 4). The kainate/AMPA receptor–specific antagonists CNQX (100 μM) and DNQX (100 μM) did not protect against hypoglycemic damage in either hippocampal or septal cultures. APV, DGG, CNQX, and DNQX alone had no significant effect on neuronal survival in cultures maintained in

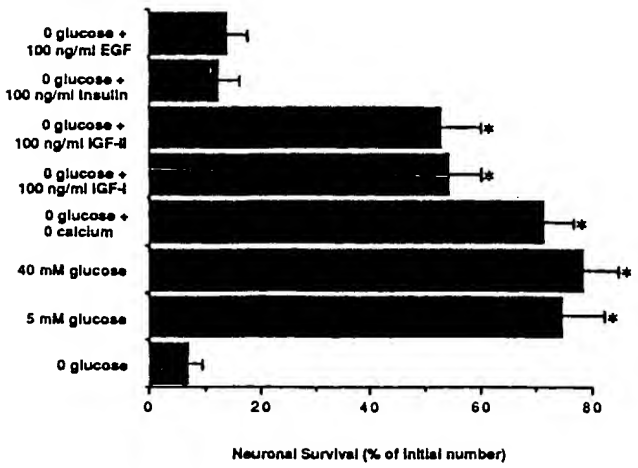


Figure 3. Evidence supporting a direct action of IGF-I and IGF-II in rat hippocampal neurons. Non-neuronal cell division was halted by a 3 d exposure to 10 μM Ara-C. Cultures were then exposed to a growth factor for 24 hr followed by an 18 hr exposure to medium lacking glucose. Values represent the mean and SEM of determinations made in three or four separate cultures. *, $p < 0.001$ compared with 0 glucose alone, 0 glucose + insulin, and 0 glucose + EGF.

glucose-containing medium (data not shown; cf. Mattson et al., 1988; Facci et al., 1990).

Effects of calcium channel blockers and kinase inhibitors on hypoglycemic neuronal damage

Calcium influx through voltage-sensitive channels (Siesjo et al., 1988) and overactivation of protein kinases (Favaron et al., 1990; Mattson, 1991) have been implicated in ischemic/excitotoxic neuronal damage. Previous work indicated that blockers of L-type calcium channels could protect neurons against excitotoxicity (Weiss et al., 1990). It was therefore of interest to determine whether voltage-sensitive calcium channels and/or calcium-dependent protein kinases played a role in glucose deprivation–induced neuronal damage. The dihydropyridine nifedipine (100 μM), and verapamil (10 μM), did not protect hippocampal neurons against death caused by glucose deprivation (Fig. 5). Similarly, two other L-type calcium channel blockers, diltiazem (10 μM) and nimodipine (20 μM), were ineffective in protecting hippocampal neurons against hypoglycemic damage (data not shown). These concentrations of calcium channel blockers did not significantly affect neuronal survival in hippocampal cultures incubated in the presence of glucose ($n = 4$ separate cultures). Similar results were obtained in septal cultures with these calcium channel blockers (data not shown). Taken together with the data above, these results indicated that calcium influx was responsible for the hypoglycemic neuronal damage, and that the damaging calcium influx occurred through the NMDA receptor channel and/or through non-L-type channels.

In order to determine whether calcium/calmodulin-dependent protein kinases were involved in the hypoglycemic damage, we employed calmodulin inhibitors that had previously been shown to protect neurons in several different paradigms of neuronal death (Rich and Hollowell, 1990; Mattson, 1991). Flunarizine (10 μM) did not afford significant protection against hypoglycemic damage (Fig. 5). Two other calmodulin inhibitors, lidoflazine (10 μM) and trifluoperazine (100 nM), also did not protect neurons against hypoglycemic injury (data not shown). Since recent evidence indicated that overactivation of protein kinase C (PKC) can result in neurodegeneration (Favaron et al., 1990; Mattson, 1991), we determined whether the PKC inhibitor H-7 would modify hypoglycemic damage. H-7 (5 μM) did not prevent neuronal damage after 18 hr of glucose deprivation in hippocampal cell cultures (Fig. 5). Flunarazine, lidoflazine, and H-7, at the same concentrations as were added to glucose-deprived cultures, did not significantly affect neuronal survival in hippocampal cultures incubated in the presence of glucose; however, trifluoperazine did cause significant neuronal degeneration (data not shown). These data indicate that blockade of calcium/calmodulin-dependent kinases and PKC will not prevent glucose deprivation-induced neuronal death.

Table 2. IGF-I and IGF-II prevent the increase in intraneuronal free calcium caused by glucose deprivation

	Intraneuronal calcium concentration (nM)	
	Hippocampus	Septum
0 glucose	346 ± 23	320 ± 20
5 mM glucose	91 ± 13*	84 ± 9*
20 mM glucose	81 ± 9*	84 ± 8*
40 mM glucose	89 ± 10*	80 ± 10*
0 glucose + 100 ng/ml IGF-I	68 ± 8*	78 ± 9*
0 glucose + 100 ng/ml IGF-II	72 ± 12*	83 ± 7*
0 glucose + 100 ng/ml EGF	265 ± 26	324 ± 17
0 glucose + 0 $[Ca^{2+}]_o$	82 ± 10*	79 ± 8*

Cultures were incubated in the presence of growth factors for 24 hr prior to exposure to glucose-free medium. Intraneuronal calcium levels were measured after 14–16 hr of incubation in the indicated conditions. Values represent the mean ± SEM of determinations made in 20–40 neurons.

* $p < 0.001$, as compared with cultures maintained in 0 glucose or 0 glucose + EGF.

Discussion

An increasing number of growth factors are being identified that protect central neurons against environmental insults. Previous work demonstrated protective effects of basic fibroblast growth factor (bFGF) and NGF against physical, ischemic, and/or excitotoxic damage (Hefti et al., 1985; Anderson et al., 1988; Mattson et al., 1989; Cheng and Mattson, 1991). The present data demonstrated the potent protective effect of IGFs against hypoglycemic damage in hippocampal and septal cell cultures. Furthermore, we provided evidence that the neuroprotective action of IGFs results from their ability to stabilize neuronal calcium homeostasis. Since central neurons are particularly vulnerable to ischemic insults, these data suggest that IGF may play a neuroprotective role under conditions of reduced energy supply.

The protective effects of IGFs and insulin against glucose deprivation were concentration dependent and specific. Both IGF-I and IGF-II were effective in reducing neuronal death at concentrations 1–3 ng/ml (approximately 150–450 pM), with half-maximally effective concentration of approximately 20 ng/ml (approximately 3 nM). The dose–response curves for the two IGFs were remarkably similar. In contrast, insulin was only effective in protecting neurons against hypoglycemic damage when administered at levels 100–1000-fold greater than the IGFs (1–3 μg/ml; approximately 200–500 nM). These data are consistent with the possibility that the protective effect of insulin

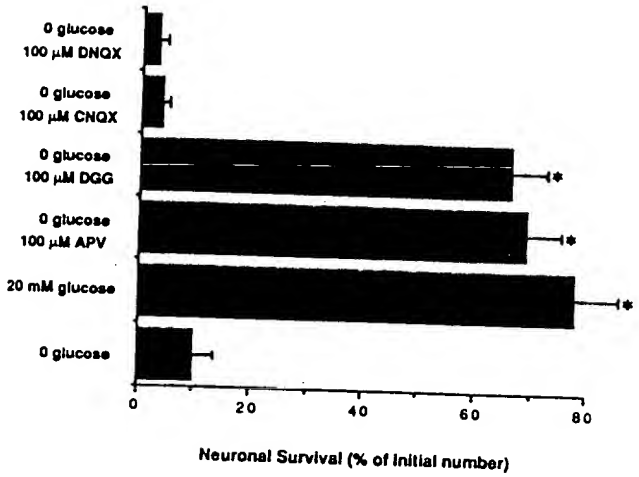


Figure 4. Effects of NMDA and non-NMDA receptor antagonists on hippocampal neuronal death due to glucose deprivation. Cultures were exposed to the indicated treatments and neuronal survival was assessed 18 hr following the onset of glucose deprivation. APV is an NMDA receptor–specific antagonist, CNQX and DNQX are non-NMDA receptor–specific antagonists, and DGG is a broad-spectrum glutamate receptor antagonist. Values represent the mean and SEM of determinations made in three or four separate experiments. *, $p < 0.001$ compared to 0 glucose, 0 glucose + CNQX, and 0 glucose + DNQX.

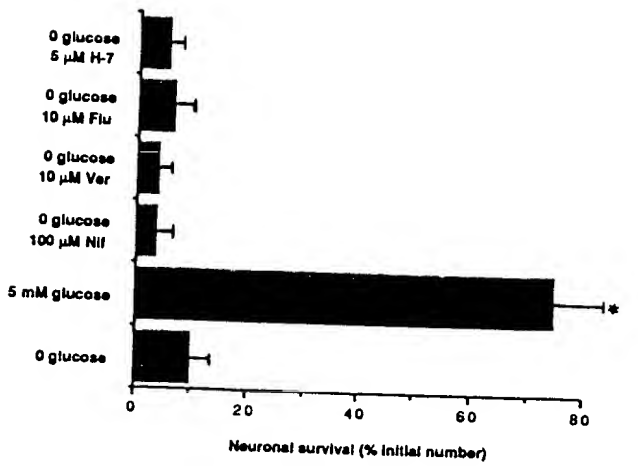


Figure 5. Effects of calcium channel blockers and kinase inhibitors on hippocampal neuronal death resulting from glucose deprivation. Inhibitors were included in the culture medium during an 18 hr period of glucose deprivation. Inhibitors were nifedipine (*Nif*), verapamil (*ver*), flunarizine (Flu), and H-7. Values represent the mean and SEM of determinations made in three or four separate cultures. Nifedipine, verapamil, flunarizine, and H-7 did not protect against the neuronal death ($p > 0.05$).

was mediated by IGF-I receptor since previous studies have demonstrated an approximately 1000-fold lower affinity of insulin (as compared to IGFs) for IGF-I receptor (Ullrich et al., 1985, 1986). Since insulin does not appear to bind to IGF-II receptor (Baskin et al., 1988), the growth or protective effects of insulin on CNS cells may be due to its binding to the IGF-I receptor. Autoradiographic studies indicated that there are IGF-I and IGF-II receptors, but no insulin receptors, in the hippocampus (Bohanon et al., 1988; Lesniak et al., 1988). IGF-I receptors are concentrated in strata radiata and oriens, particularly in region CA3, suggesting that they may be present on pyramidal neurons. *In situ* hybridization studies indicated that insulin and IGF-I receptor mRNAs are present in hippocampus (Baron-Van Evercooren et al., 1991). We found that IGF-I and IGF-II were equipotent in protecting neurons against hypoglycemia. When taken together with the fact that IGF-I has a higher affinity for IGF-I receptors than does IGF-II (and vice versa), our data suggest that hippocampal and septal neurons have both IGF-I and IGF-II receptors.

Rat hippocampal cultures contain essentially all non-cholinergic neurons, whereas septal cultures contain a large population of ch linergic neurons (Hefti et al., 1989). Previous studies showed that IGFs and insulin elevated ChAT activity in septal cultures and dopamine uptake in mesencephalic cultures (Knusel et al., 1990). No data were previously available concerning actions of IGFs and insulin in hippocampal neurons. Our data indicate that IGFs are likely to influence a rather large number of neuronal types in the brain. As with the IGFs, bFGF has been shown to support cell survival and neurite outgrowth in cultured neurons (including cholinergic and non-cholinergic) from various brain regions (Morrison et al., 1986; Walicke et al., 1986; Unsicker et al., 1987; Hatten et al., 1988; Walicke, 1988). Intracerebral administration of bFGF prevents degenerative changes of lesioned cholinergic neurons of the basal forebrain (Anderson et al., 1988). In contrast to IGFs and FGF, the biological actions of NGF in the CNS seem to be limited to certain populations of primarily cholinergic neurons (Thoenen et al., 1987; Hefti et al., 1989; Snider and Johnson, 1989). On the other hand, we recently found that NGF and bFGF protected both rat hippocampal and human cortical neurons against hypoglycemic damage (Cheng and Mattson, 1991), indicating that NGF, in addition to actions on peripheral neurons and central cholinergic neurons, can also directly affect central non-cholinergic neurons. Taken together, the available data indicate that IGF-I and IGF-II, as well as bFGF and probably NGF, affect both cholinergic and non-cholinergic central neurons. The extent to which the signal transduction systems for these different growth factors are different or overlapping remains to be determined.

Insulin and IGFs are known to affect glial cells (Lenoir and Honegger, 1983; Avola et al., 1988). Glial cells, in turn, are known to provide trophic support for neurons (Mattson and Rychlik, 1990). In the present study, however, treatment of the cultures with Ara-C to prevent cellular proliferation only slightly reduced the neuroprotective effects of IGFs. This suggests that the neuroprotective effects of IGFs were mainly the results of direct actions on the hippocampal and septal neurons, although a role for glia was not completely ruled out.

The mechanism of hypoglycemic neuronal damage is not completely understood, but appears to involve a loss of neuronal calcium homeostasis. In support of this mechanism, we found that removal of extracellular calcium prevented hypoglycemic damage. Contributing to the loss of calcium homeostasis was calcium influx triggered by activation of NMDA receptors since APV, a specific blocker of NMDA-type calcium channels, significantly reduced hypoglycemic damage. This possibility is consistent with previous data obtained in mouse cortical and rat cerebellar cultures wherein hypoglycemic damage was reduced by NMDA receptor antagonists (Monyer et al., 1989; Facci et al., 1990). Non-NMDA receptor antagonists (CNQX and DNQX) did not reduce hypoglycemic neuronal damage in the present study, indicating that activation of non-NMDA recep-

tors is probably not necessary for hypoglycemic damage. In addition, dihydropyridine blockers of L-type calcium channels (nimodipine and nifedipine), as well as verapamil and diltiazem did not reduce hypoglycemic damage, indicating that calcium influx through the L-type channel was not a major factor contributing to the loss of calcium homeostasis. Taken together, these findings suggest that calcium influx through the NMDA receptor channel was a major contributor to the degenerative effects of glucose deprivation. However, we cannot yet rule out the possibility that calcium influx through non-L-type calcium channels plays a role in hypoglycemic neuronal damage, since a substantial portion of high-threshold calcium current is resistant to dihydropyridines and ω-conotoxin (Regan et al., 1991).

Both IGF-I and IGF-II prevented hypoglycemia-induced loss of neuronal calcium homeostasis. The specific system(s) for calcium homeostasis affected by IGFs is not clear. Previous work provided evidence that neuronal growth factors can stabilize neuronal calcium homeostasis. For example, NGF influenced the expression of calcium channels and calcium-extruding systems in PC12 cells (Takahashi et al., 1985; Chalazonitis et al., 1987; Masiakowski and Shooter, 1988; Streit and Lux, 1990). In addition, bFGF protected cultured rat hippocampal neurons against excitatory amino acid neurotoxicity (Mattson et al., 1989). The protective effect of bFGF is mediated at least in part by a suppression of the expression of an NMDA receptor protein by this growth factor (Mattson et al., 1991; Michaelis et al., 1991). In addition, bFGF may enhance the ability of neurons to reduce intracellular calcium levels following an excitatory challenge (Mattson and Rychlik, 1990). In a recent related study we found that NGF and bFGF protected rat hippocampal and human cerebral cortical neurons against neuronal damage caused by glucose deprivation (Cheng and Mattson, 1991). In the latter study we found that both NGF and bFGF prevented the loss of neuronal calcium homeostasis that normally mediated hypoglycemic damage. Growth factors may also stabilize intracellular calcium levels in peripheral neurons. For example, in cultured sympathetic neurons NGF appears to influence neuronal systems for calcium homeostasis and these effects of NGF are correlated with its trophic action (Koike and Tanaka, 1991). Taken together, the available data suggest that a general feature of growth factor action may be to stabilize neuronal intracellular free calcium levels.

Overactivation of protein kinases has been suggested to be involved in the neuronal damage that occurs in a number of neurodegenerative conditions. For example, ischemic brain damage is associated with altered PKC activity (Louis et al., 1988), and administration of a PKC inhibitor was found to reduce ischemic damage (Joo et al., 1989). Overactivation of calcium/calmodulin-dependent kinase(s) and PKC have been implicated in the neurofibrillary degeneration that occurs in Alzheimer's disease and related disorders (Mattson, 1990, 1991). In the present study, calmodulin inhibitors (flunarizine, lidoflazine, and trifluoperazine) and the PKC inhibitor H-7 did not protect hippocampal or septal neurons against hypoglycemic damage. Thus, we were not able to provide evidence supporting a role for calmodulin-dependent protein kinases or PKC in hypoglycemic damage. These data suggest that these calcium-regulated kinases may not be involved in hypoglycemic damage. However, since we did not directly assess kinase activity, we cannot rule out the possibility that the kinase inhibitors used did not completely block kinase activities. Clearly, further work will be required to understand the specific events triggered by glucose deprivation that lead to a loss of neuronal calcium homeostasis and cell death.

The protective effects of IGFs against hypoglycemic damage demonstrated here are consistent with the possibility that IGFs play a neuroprotective role *in vivo*. In the developing nervous system IGFs may play a role in determining which neurons survive during the period of naturally occurring neuronal death, and in the process of synaptic organization. From a pathological standpoint, IGFs may also play a role in preventing neuronal death. The hippocampus and septal area are brain regions that are particularly vulnerable in acute (e.g., stroke) and chronic (e.g., Alzheimer's disease) neurodegenerative disorders. The present data may therefore have implications for approaches to preventing neuronal damage in these disorders.

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Insulin-like growth factor I protects and rescues hippocampal neurons against β-amyloid- and human amylin-induced toxicity

(neurorescuing effect/Alzheimer disease)

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ABSTRACT **Insulin-like growth factors (IGF-I and IGF-II) are well known trophic factors and their specific receptors are uniquely distributed throughout the brain, being especially concentrated in the hippocampal formation. IGFs possess neurotrophic activities in the hippocampus, an area severely affected in Alzheimer disease. These data, together with the evidence that β-amyloid (Aβ)-derived peptides likely play an important role in the neurodegenerative process observed in Alzheimer disease, led us to investigate if IGFs could be neuroprotective to hippocampal neurons against toxicity induced by amyloidogenic derivatives. Exposure of rat primary hippocampal neurons to different concentrations of $A\beta_{25-35}$, $A\beta_{1-40}$, $A\beta_{1-42}$, and human amylin produced marked toxicity, while similar concentrations f two control Aβ peptides—reverse ($A\beta_{40-1}$) and scrambled sequence ($A\beta_{25-35}$)—and rat amylin failed to exhibit any significant effect on neuronal survival. IGF-I (10–100 nM) significantly protected hippocampal neurons against neurotoxicity induced by Aβ derivatives and human amylin. The homolog IGF-II was also effective although less potent than IGF-I suggesting the involvement of a typical IGF-I receptor in the observed neuroprotective effect. Most interestingly, IGF-I (10–100 nM) was even able to rescue neurons pre-exposed (up to 4 days) to amyloidogenic peptides. Other neurotrophic factors are reported to lack such rescuing abilities. These results suggest that IGF-I may have unique properties as a potent neuroprotective and neurorescuing agent against amyloid-related neurotoxicity.**

β-Amyloid ($A\beta_{1-40}$, $A\beta_{1-42}$) is believed to play a role in the neurodegenerative process occurring in Alzheimer disease (AD) (1, 2). This protein is found deposited in extracellular neuritic plaques, one of the hallmarks of the AD brain along with the presence of neurofibrillary tangles and cell losses in various regions, including the basal forebrain (3). Aβ is derived proteolytically from a larger transmembrane protein, the amyloid protein precursor, which is expressed widely throughout the brain (4, 5). The direct, toxic properties of Aβ-related fragm nts ($A\beta_{1-40}$, $A\beta_{1-42}$, $A\beta_{25-35}$) in cultured rat and human n urons and in *in vivo* are well established (6–8), although the mechanism of action involved remains to be established.

Insulin-like growth factors (IGF-I and IGF-II) play an important role in the normal development and maintenance of the cellular integrity of the organism, including the central n rvous system (9). Both trophic factors are selectively localized in the brain and their specific receptors are uniquely distributed in various neuroanatomical regions, being especially concentrated in the hippocampal formation (10). The IGF-I receptor is composed of two α chains where the ligand binds and two β chains possessing a tyrosine kinase domain (11). In contrast, the IGF-II receptor is made of a single transmembrane segment containing a binding site for IGF-II and another for mannose-6-phosphate residues. Both receptors bind specifically to their cognate ligands but can also recognize the other with lower affinity. We have recently shown that cultured hippocampal neurons are highly enriched with IGF-I and IGF-II receptors each being differentially internalized (12), and serving distinct functions (13). Earlier studies have shown that IGFs possess neurotrophic activities in the hippocampus (14–16), an area severely affected in AD. Interestingly, it was also observed that the levels of IGF-I binding sites are significantly increased in cortical areas of AD brains (17). It is unclear if these increases in IGF-I receptors represent a protective and/or compensatory mechanism against neuronal losses.

Considering the broad actions of IGFs on the maintenance of normal cellular functions and the presence of high levels of IGF receptors in the hippocampus, we investigated the potential neuroprotective effects of IGFs against Aβ-induced toxicity in rat hippocampal neurons. Human amylin, which also has amyloidogenic properties (18), was also studied for comparison. Our results show that IGF-I is able not only to protect neurons against Aβ-induced toxicity but even to rescue them up to a few days following exposure to Aβ derivatives and human amylin. Preliminary results were presented in abstract form (58).

MATERIALS AND METHODS

Materials. Different fragments of Aβ peptides including $A\beta_{25-35}$ lot no. ZM501; $A\beta_{1-28}$ lot no. ZK792; $A\beta_{1-40}$ lot no. ZM365; $A\beta_{40-1}$ lot no. ZL511; $A\beta_{1-42}$ lot no. ZN052 (Figs. 1–3) and $A\beta_{1-42}$ lot no. ZM823 (Figs. 4–6) were purchased from Bachem. The scrambled sequence of $A\beta_{25-35}$ was generously provided by P. Gaudreau (Notre-Dame Hospital Research Center, Montreal). Rat and human amylins were bought from Peninsula Laboratories. Recombinant human (rh)IGF-I was obtained from Genentech while hIGF-II was bought from Lilly Research Laboratories (Indianapolis). Materials used for cell culture were obtained from GIBCO/BRL. Unless stat d otherwise, all other chemicals were purchased from Sigma.

Hippocampal Neuron Cultures and Experimental Treatments. Hippocampal neuronal cells, as described earlier (12), were prepared from fetuses (embryonic day 19) obtained from time-pregnant Sprague–Dawley rats (Charles River Breeding Laboratories). Animal care was according to protocols and guidelines approved by the McGill University Animal Care Committee and th Canadian Council for Animal Car .

Following dissection, hippocampal cells were plat d at high density (7.5×10^5 cells per well) in 16-mm tissu cultur dishes coated with poly-D-lysine (10 μg/ml) under serum-free con-

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Abbreviations: Aβ, β-amyloid; AD, Alzheimer disease; FGF, fibroblast growth factor; IGF, insulin-like growth factor; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NGF, nerve growth factor; TGF, transforming growth factor.

ditions with high glucose DMEM supplemented with B27 (GIBCO). High-density cultures offer the opportunity to score cell viability by a nonsubjective, easily quantifiable enzymatic assay (see below). On the day following plating, the medium was replaced with fresh culture medium, one-third of the medium being changed again after 3 days. Experiments were performed after 6 days in culture, at which time pyramidal neurons are fully differentiated (19).

Experimental treatments that include exposure to various Aβ-related peptides, human amylin, rat amylin, IGF-I, and IGF-II, and were conducted in the N-2 supplement Hepes-buffered high glucose neurobasal medium (GIBCO). Aβ peptides were dissolved in dimethyl sulfoxide and used immediately by directly diluting to the indicated concentrations in the chemically defined experimental culture medium. Sister cultures were treated with appropriate vehicle controls and the final concentration of dimethyl sulfoxide did not exceed 0.01%. Following treatments, neurons were maintained for an additional period of 6 days and their survival was assessed by phase-contrast microscopy and quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The neuroprotective effect of IGF was evaluated by treating the cells simultaneously with IGF and different Aβ- or amylin-related peptides, while the neurorescuing action of IGF was determined by treating the cells at different days following initial exposure to Aβ- or amylin-related peptides.

MTT C lorimetric Assay. MTT is a purported indicator of the mitochondrial activity in living cells (20). Evidence suggests that Aβ-induced toxicity measured as a percentage of MTT reduction correlates with percentage release of lactate dehydrogenase (21) and it represents an excellent marker of metabolic compromise that ultimately leads to neuronal degeneration and cell death (20–22). In the present study, after 6 days of experimental treatments, the culture medium was replaced with DMEM and freshly dissolved MTT (stock: 5 mg/ml in 0.1 M PBS) was aseptically added to a final concentration of 10%. The plates were then returned into the incubator for a 3-hr period. Cells and MTT formazan crystals were solubilized by trituration in a solution of anhydrous isopropanol·HCl 0.1 N and spectrophotometrically measured at 570 nm. Survival of control vehicle-treated neuronal cells not exposed to Aβ- or amylin-related peptides was set at 100% and treated groups were represented as percentage of control values. All experiments were repeated at least with three separate batches of cultures and the means were analyzed using GraphPad (San Diego) PRISM 2.01. Results were represented as the mean ± SEM with $P < 0.01$ was considered significant. Student's t test was used to establish significance.

RESULTS

Neur nal Toxicity Induced by Different Aβ-Related Peptides. Various Aβ peptides (i.e., $A\beta_{1-40}$, $A\beta_{1-42}$, and $A\beta_{25-35}$) are found to be highly toxic to cultured rat hippocampal neurons as evidenced by concentration-dependent reduction in MTT values (Fig. 1). Of these three fragments, $A\beta_{1-42}$ was the most potent to affect cell survival. In contrast, $A\beta_{1-28}$ and the two control peptides, $A\beta_{40-1}$ (reverse sequence) and $A\beta_{25-35}$ (scrambled sequence), were not toxic and did not affect th survival of neurons thus confirming the specificity to th bserved toxic effects.

Evidence suggests that pre-aggregated Aβ peptide is highly toxic to a variety of cultured neurons. To compare the relative potency f pre-aggregated Aβ peptide, we aged $A\beta_{25-35}$ (1 mM) in the incubator for 4 days at 37°C prior to the experiment (23). Th aged $A\beta_{25-35}$ was also n urotoxic (data not sh wn), although not more effective than the freshly solubilized peptide. This is likely due to the fact that our experimental procedures include xposure of cultured neurons to Aβ fragments for a six-day period at 37°C, aggregation likely occurring during this period, hence artificial aging not being required.

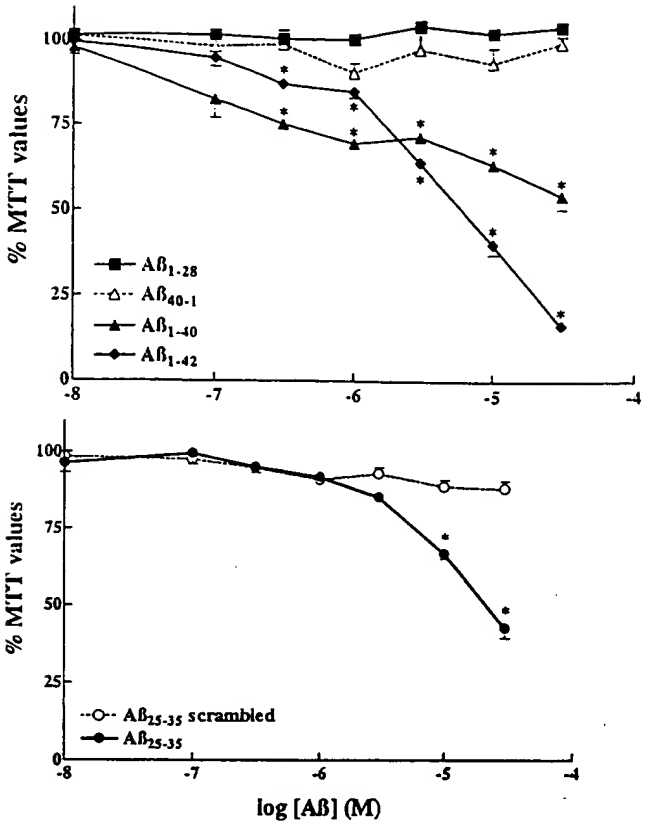

FIG. 1. Neuronal toxicity induced by different Aβ-related peptides. Rat primary hippocampal neurons were treated for 6 days with different concentrations of $A\beta_{1-28}$, $A\beta_{1-40}$, the control reversed sequence $A\beta_{40-1}$, $A\beta_{1-42}$ (*Upper*) and $A\beta_{25-35}$, and its control scrambled sequence ($A\beta_{25-35}$ scrambled) (*Lower*). *, $P < 0.01$ as control treatments.

Neuroprotective Effects of IGF-I and IGF-II Against Aβ-Induced Toxicity. As shown in Fig. 2, increasing concentrations of IGF-I and IGF-II are able to protect hippocampal neurons against $A\beta_{25-35}$-induced toxicity (30 μM $A\beta_{25-35}$; 44% of control values). IGF-I is found to be more potent than IGF-II (Fig. 2). When treated alone with either IGF-I or IGF-II, the survival of neurons is not found to be significantly affected (Fig. 2, top lines).

Neurorescuing Effects of IGF-I Against Aβ-Induced Toxicity. Fig. 3 shows that IGF-I at 100 nM is able to significantly protect neurons against toxicity induced by 30 μM $A\beta_{25-35}$ even if added up to 3–5 days post-Aβ treatments. Neurons incubated with Aβ alone were highly affected with MTT values down to 34% of control. When the cells were incubated with IGF-I immediately or up to 2 days after Aβ exposure, IGF-I rescued neurons with MTT values being significantly increased between 68–72% of controls. At subsequent days (3–5), the rescuing effect was still significant but less evident indicating that neurons are too affected to fully benefit from IGF-I rescuing properties. At the same concentration, IGF-II had a slight protective effect when incubated simultaneously with Aβ but failed to demonstrate any rescuing abilities (Fig. 3).

Morphological Features of Neurons Exposed to $A\beta_{1-42}$ and/or IGF-I. Fig. 4 summarizes the morphological features of exposure to IGF-I, Aβ, and their combination (simultaneously or post-Aβ treatment). $A\beta_{1-42}$, at a concentrati n of 5 μM, induced degeneration with shrinkage of cell soma, neuronal clustering, and debris. IGF-I added simultaneously at 10, 30, and 100 nM was clearly neuroprotective (Fig. 4, second row). M reover, IGF-I (30 nM) was abl to rescue neurons against $A\beta_{1-42}$ toxicity even when added 1, 2, or 4 days later (Fig. 4, bottom row).

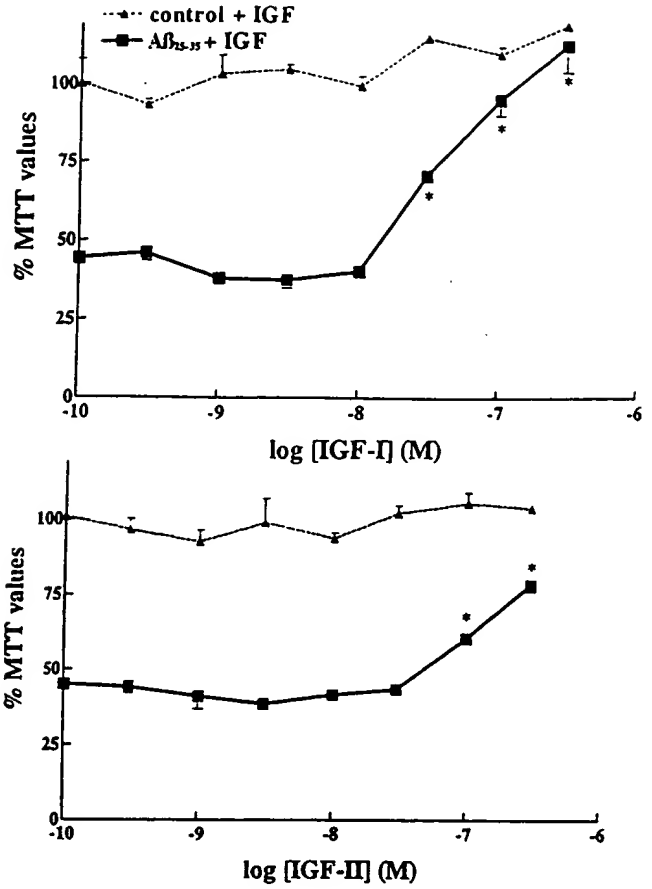

FIG. 2. Neuroprotective effect of IGF-I (*Upper*) and IGF-II (*Lower*) against $A\beta_{25-35}$-induced toxicity in rat primary hippocampal neurons. $A\beta_{25-35}$ alone (30 μM), induced a marked loss in MTT values down to 44% of controls. Concentration-dependent neuroprotective effects of IGF-I and IGF-II are observed against $A\beta_{25-35}$ toxicity when the trophic factors are added simultaneously with $A\beta_{25-35}$. Increasing concentrations of IGFs, by themselves, failed to have significant effects in control cultures (dashed lines). IGF-I was clearly more effective than IGF-II. *, $P < 0.01$ as Aβ-treated neurons.

Neuroprotective/Neurorescuing Effects of IGF-I Against Amylin-Induced Toxicity. Human amylin was highly toxic to cultured hippocampal neurons as shown by decreased concentration-dependent MTT values (Fig. 5). In contrast, rat amylin was unable to induce toxicity, in accordance with an earlier report (18). Fig. 6 *Upper* shows that increasing concentrations f IGF-I were able to protect neurons against toxicity induced by 30 μM human amylin. Neurons incubated with human amylin alone (35 μM) were highly affected with MTT values down to 26% of control (Fig. 6 *Lower*). IGF-I (100 nM) was able to significantly rescue neurons for up to 4 days after treatment with human amylin, with important rescuing abilities observed after 2 days. At subsequent days, the rescuing ability of IGF-I was still significant but less evident (Fig. 6).

DISCUSSION

The major finding of the present study relates to the neuroprotectiv and neurorescuing properties of IGF-I against Aβ-induced toxicity. While various neurotrophins and neurotrophic factors (24) have been shown to block the toxic effect of Aβ derivatives *in vitro* and *in vivo*, th rescuing action of IGF-I is, to our knowledg , unique. Indeed, w observed that th incubation of IGF-I was able to rescue primary rat hippocampal neurons pre-exposed to Aβ for up to 4–5 days. This is particularly interesting in the clinical context and

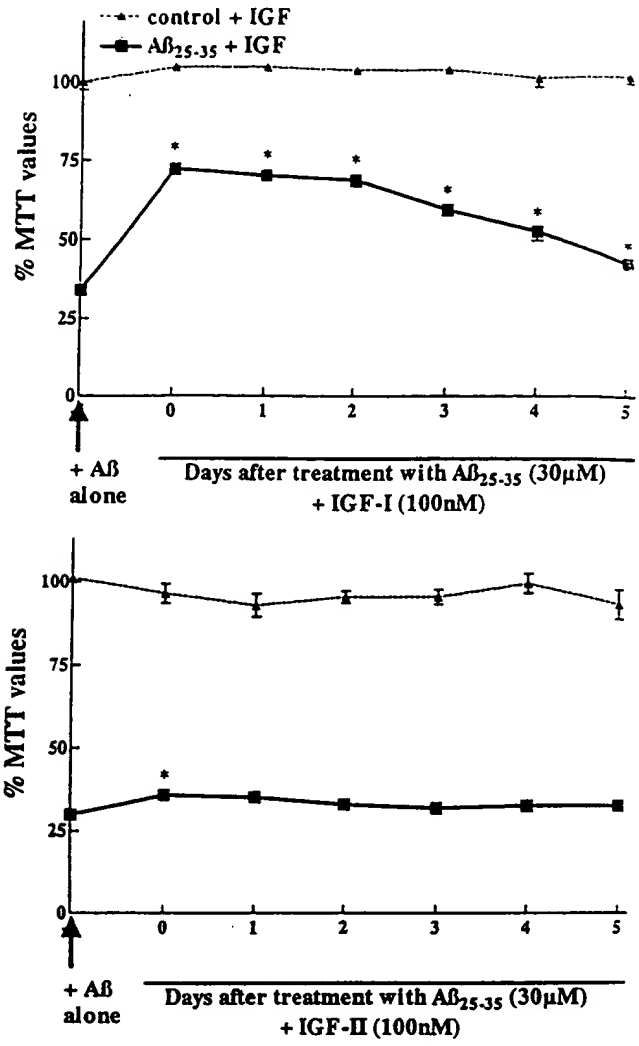

FIG. 3. Effect of IGF-I (*Upper*) and IGF-II (*Lower*) on the survival of primary hippocampal neurons previously exposed to $A\beta_{25-35}$. Neurons were first incubated with 30 μM $A\beta_{25-35}$ and at different days posttreatment, 100 nM of IGF-I or IGF-II was added. Neurons incubated with Aβ alone showed marked loss in MTT values down to 40% of controls. Most interestingly, IGF-I can rescue neurons from Aβ toxicity even up to 3 days posttreatment (*Upper*). At later stages, the rescuing effect of IGF-I is not as evident (although still significant) likely because neurons are too affected to benefit from the action of IGF-I. At the same concentration, IGF-II showed protective action when incubated simultaneously with Aβ but did not show any significant rescuing properties (*Lower*). *, $P < 0.01$ Aβ-treated neurons.

suggests that the mechanism of action of Aβ-induced toxicity involved a rather long process allowing for various pharmacological interventions such as the use of IGF-I, its mimetics, or molecules activating the IGF-I receptor signaling pathway.

It is now rather well established that various Aβ derivatives, especially in their aggregated forms, are toxic to many cell types, including neurons both *in vitro* and *in vivo* (8, 25, 26). We confirmed and extended these findings in the present study. The main amyloidogenic components of the neuritic plaques are the $A\beta_{1-42}$ and $A\beta_{1-40}$ fragments (1, 27). $A\beta_{1-40}$, $A\beta_{1-42}$ fragments and the $A\beta_{25-35}$ peptide (which contains the active toxic domain) are highly toxic to rat primary hippocampal neurons as exemplified by the reduction in MTT values and the altered morphological features of the culture. In contrast, the nonamyloidogenic fragment $A\beta_{1-28}$ and the controls (including the random sequence f the $A\beta_{25-35}$ and the reverse sequence peptide, $A\beta_{40-1}$) did not affect neuronal survival. These results confirm that the neurotoxic properties of Aβ derivatives are highly sequ nce

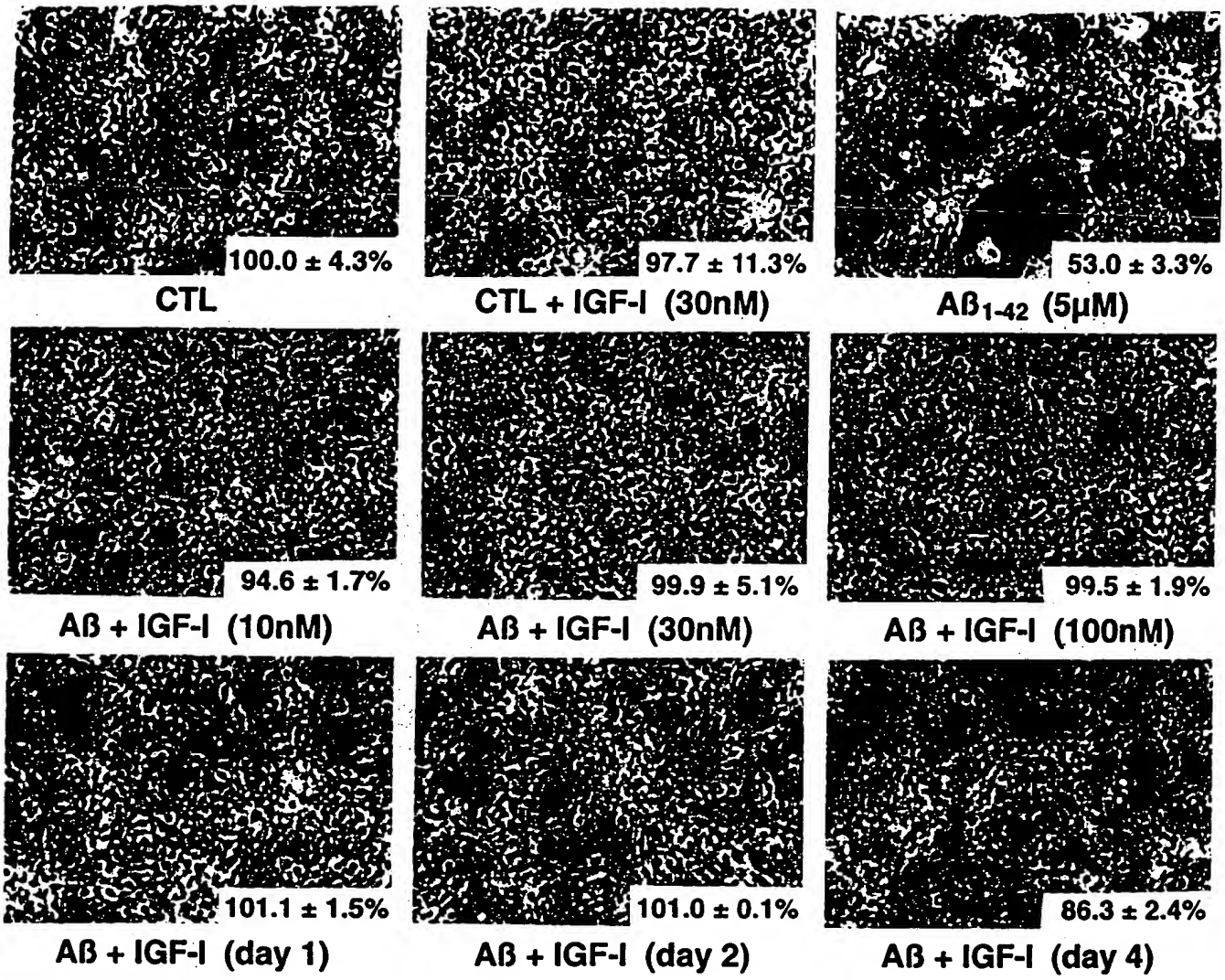

FIG. 4. Photomicrographs showing $A\beta_{1-42}$ (5 μM)-induced cytotoxicity and neuroprotective effects of IGF-I. Phase contrast micrographs of primary hippocampal neurons exposed for 6 days with different treatments. MTT values are provided (lower right corner) to better assess the correlation with morphological changes. The upper left corner is control (CTL) representing neurons incubated with vehicle only, and MTT value is being fixed at 100%. The subsequent panel is the control cells (CTL) + IGF-I (30 nM) and then $A\beta_{1-42}$ (5 μM) by itself. The second row pictured neurons incubated with $A\beta_{1-42}$ and different concentrations of IGF-I (10, 30 and 100 nM). The bottom row shows neurons treated with $A\beta_{1-42}$ and with IGF-I (30 nM) added subsequently and at different days (days 1, 2, and 4). The numbers indicated in the bottom right corner are the MTT values collected from these cells. (Bar = 50 μM.)

dependent and possibly related to their amphiphilic nature. This hypothesis is supported further by the fact human, but not rat, amylin was found to be neurotoxic in various models (18, 28), including ours. Human amylin, which is present in the brain (28), is a highly amphiphilic peptide that has a high tendency to aggregate in contrast to rat amylin that is nontoxic (29).

The initial event leading to Aβ-induced toxicity is unknown at present but may involve plasma membrane receptors for advanced glycation end products (RAGE; ref. 30), class A scavenger receptor-related proteins (31), certain G-proteins (32), heparan sulfate (33, 34), and α_2-macroglobulin extracellular domains (35). Whether IGF-I can directly interact with these various receptor sites to block Aβ-induced toxicity remains to be established. Moreover, we cannot rule out the possibility that IGF-I could interfere with the aggregation of Aβ or human amylin. Preliminary experiments, how ver failed t provide evidence for differential fibril f rmation in the presence ($A\beta_{25-35}$; 30 μM plus IGF-I, 100 nM) or absence (Aβ alone) f IGF-I (unpublished results).

We bserved that both IGF-I and IGF-II wer able to protect rat hippocampal neurons against Aβ-induced neurotoxicity. However, IGF-I was clearly more potent than IGF-II. Additionally, IGF-II, at the concentration tested (100 nM), was unable to rescue neurons previously exposed to $A\beta_{25-35}$. Taken together, these results suggest the involvement of the IGF-I receptor subtype known to be activated by both IGF-I and IGF-II, the latter with markedly lower potency.

Other trophic factors have been reported to protect neurons against various types of insults. For example, nerve growth factor (NGF) (36), basic fibroblast growth factor (bFGF) (37), and transforming growth factors (TNF) α and β (38) have been shown to protect cultured hippocampal neurons against Aβ-induced toxicity. However, none of these trophic factors were effective post-Aβ-treatment hence lacking neurorescuing properties. Interestingly, TGFβ_1 and TGFβ_2, but not TGFβ_3, wer apparently able to have a slight protective effect in neurons pre-exposed to $A\beta_{25-35}$ for 24 hr while such effect was not observed with TNF, NGF, aFGF, or bFGF (39). The rescuing ability of TGFβ_1 and TGFβ_2 was not as pronounced as the one observed with IGF-I and was not as effective against longer exposures to $A\beta_{25-35}$. Moreover, preliminary results with TGFβ (100 nM) and NGF (10 to 1,000 nM) failed to reveal any rescuing abilities of these two factors against toxicity induced by $A\beta_{25-35}$ in our model (unpublished results). Taken

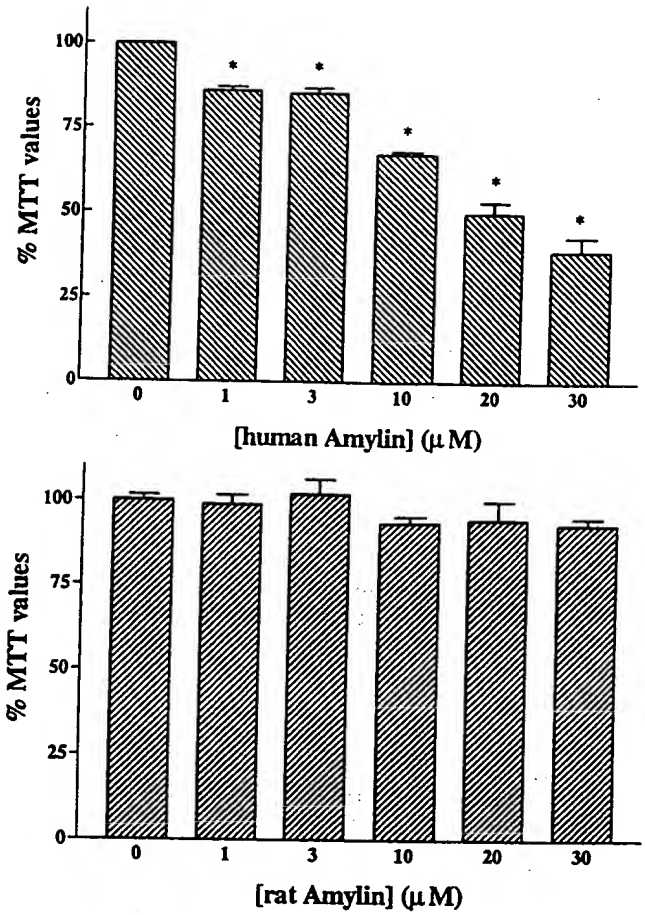

FIG. 5. Neuronal toxicity induced by human (*Upper*) and rat (*Lower*) amylin. Rat primary hippocampal neurons were treated for 6 days with different concentrations of amylins. Human amylin induced concentration-dependent marked losses in MTT values down to 38% of controls. Rat amylin did not induce significant changes in MTT values at the concentrations used. *, $P < 0.01$ as control treatments.

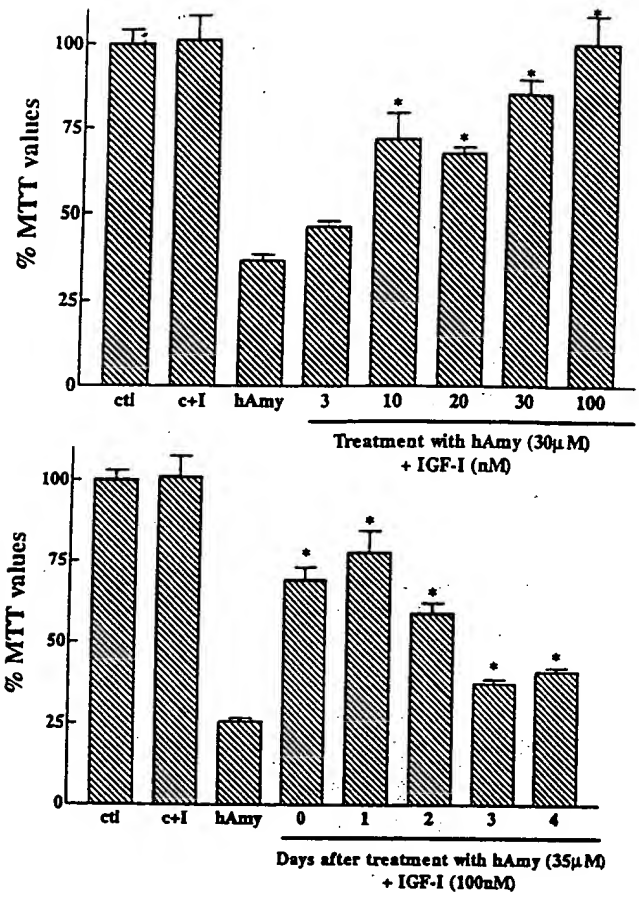

FIG. 6. Neuroprotective effects of IGF-I against human amylin-induced toxicity in rat primary hippocampal neurons. (*Upper*) Human amylin (hAmy) (30 μM) by itself, induced a marked loss in MTT values down to 36% of controls. Concentration-dependent neuroprotective effects of IGF-I are observed when added simultaneously with human amylin. IGF-I (100 nM), by itself, failed to have a significant effect in control cultures (C+I). (*Lower*) The effect of IGF-I on neuronal survival previously exposed to human amylin. Neurons were first incubated with 35 μM human amylin and at different days posttreatment, 100 nM of IGF-I was added. IGF-I can significantly rescue neurons from human amylin toxicity even up to 4 days posttreatment. *, $P < 0.01$ as hAmy treated neurones.

together, it appears that the neuroprotective/neurorescuing abilities of IGF-I against Aβ- and human amylin-induced toxicities observed in the present study are rather unique.

The mechanism of action involves in neuroprotective and especially neurorescuing properties of IGF-I against Aβ-induced toxicity remains to be clarified. The activation of the IGF-I tyrosine kinase receptor induces protein phosphorylation followed by a cascade of intracellular events that include the activation of insulin receptor substrates 1 and 2, and phosphoinositide 3-kinase, phosphotyrosine phosphatases, S6 kinase, Ras-mitogen-activating protein kinase and transcription factors leading to alterations in Ca^{2+} storage and mobilization, and mitochondrial respiration (40, 41). It has been proposed that Aβ-induced toxicity is due to free radicals production/oxidative stress (2, 42) and/or increased free intracellular Ca^{2+} levels (43) leading to necrosis and cell death (21). The toxic properties of Aβ-derivatives could also relate to their abilities to stimulate apoptotic genes/cellular events (7, 44, 45). Accordingly, IGF-I could interfere at different stages of the necrotic or apoptotic pathway to block and even rescue neurons against Aβ-induced cell death. Interestingly, IGF-I has already been shown to block programmed cell death in various models. For example, IGF-I and th IGF-I receptor prevent topoisomerase I inhibitor etoposide-induced apoptosis in 3T3 cells (46), inhibit interleukin-3-dependent cell death in various cell lines (47), inhibit apoptosis induced by either TNFα, radiation and dysregulated c-myc expression (48) and protect differentiated PC12 cells against cell death following NGF withdrawal (49). IGF-I has also been shown to prevent apoptosis associated with K^+-deprivation in cerebellar granule neurons, other factors tested including aFGF, bFGF, platelet-derived growth factor, and neurotrophin 3 being inactive (49). Similar protective effects were observed in a hybrid dopaminergic cell line against oxidation and hypoglycemia-induced cell death, IGF-I being more potent than bFGF, epidermal growth factor, and NGF (50). More recently, it was demonstrated that IGF-I (and supraphysiologic concentrations of insulin) activates a phosphoinositide 3-kinase and the serine-threonine protein kinase Akt to promote the survival of rat primary cerebellar neurons after induction of apoptosis by serum deprivation (51). It would be of interest to investigate if this pathway is also involved in the neuroprotective/neurorescuing properties of IGF-I observed in the present study. *In vivo*, IGF-I has been shown to reduce neuronal cell losses observed following hypoxic-ischemic insults (15, 52) and is beneficial in the treatment of amyotrophic lateral sclerosis patients (53). Hence, IGF-I by acting on necrotic and/ r apoptotic cellular events could protect and more importantly rescue neurons against Aβ- and human amylin-induced toxicity.

It is well established that insulin-like family members are critically involved in maintenance of body homeostasis. Aging is associated with changes in basic functions among which glucose metabolism is central to the nervous system. In aged rats and

humans, poor cognitive performances have been correlated with impaired glucose regulation (54). In sporadic AD cases, significant reductions in glucose utilization have been reported (55) and neuroglucopenia reduced the formation rate of ATP and acetylcholine from 54% to 47% of control values, respectively (55). Moreover, IGF-I receptor binding levels are apparently increased in affected areas of the AD brain (17), may be as an attempt to counteract energy metabolism deficits and cell losses. Interestingly, IGF-I is also known to be neuroprotective against toxicity induced by glucose deprivation (14, 50, 56) and it has been shown that exposure to subthreshold doses of Aβ derivatives render neurons more susceptible to glucose deprivation (57). The unique capacity of IGF-I to protect and rescue neurons against Aβ-induced toxicity, in parallel to its homeostatic potential to insure adequate glucose/energy metabolism exemplified even further the critical relevance of this trophic factor in normal and pathological brain functioning.

In summary, we observed that IGF-I is able to protect, and more importantly, to rescue rat hippocampal primary neurons against Aβ- and human amylin-induced toxicity. IGF-II is less potent suggesting the activation of the IGF-I receptor and the related signaling pathway. These unique properties of IGF-I, in parallel to its well known involvement in various metabolic pathways, suggest that the development of IGF-I-related mimetics could be a promising strategy toward the treatment of various neurodegenerative diseases including AD.

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Insulin-Like Growth Factor I Stimulates Oligodendrocyte Development and Myelination in Rat Brain Aggregate Cultures

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Insulin-like growth factor I (IGF-I) and high concentrations of insulin have been shown to stimulate an increase in the number of oligodendrocytes that appear in developing monolayer cultures of rat brain cells (McMorris et al., Proc Natl Acad Sci USA 83: 822–826, 1986; McMorris et al., Ann NY Acad Sci 605:101–109, 1990; McMorris and Dubois-Dalcq, J Neurosci Res 21:199–209, 1988). In the present study, we investigated whether IGF-I or insulin treatment induces a corresponding increase in the synthesis and accumulation of myelin. Aggregate cultures, established from 16-day-old fetal rat brains, were treated with either 100 ng/ml IGF-I or 5,000 ng/ml insulin and analyzed for the number of oligodendrocytes, activity of 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP), total amount of myelin, and synthesis rate of myelin proteins. Cultures treated with IGF-I beginning on day 2 after explanation contained 35–80% more oligodendrocytes and had 60–160% higher CNP activity than controls when tested on day 13, 20, or 27. By day 27, treated cultures had 35–90% more myelin than controls. Similar results were observed in response to 5,000 ng/ml insulin, a concentration at which insulin binds to IGF receptors and acts as an analogue of IGF-I. The synthesis rate of myelin proteins was measured in experiments using 5,000 ng/ml insulin. When treatment was begun at day 20 rather than day 2, cultures did not exhibit an increased number of oligodendrocytes over control during the following 4–6 days. Therefore, this paradigm was used to separate the effects of insulin-like peptides on the number of oligodendrocytes from the effects on the synthesis rate of myelin proteins and the accumulation of myelin. Cultures treated from day 20 and controls showed no difference in either the myelin yield or the synthesis rate of myelin proteins. These data show that IGF-I (and insulin at high concentrations) increases the amount of myelin produced in aggregate cultures; and that the increase appears to be due primarily to an increase in the number of oligodendrocytes in the cultures rather than to a direct increase in the rate of myelin protein synthesis or an increase in the amount of myelin synthesized per oligodendrocyte.

Key words: oligodendrocyte development, myelin synthesis, aggregate brain culture, 2′3′-cyclic nucleotide-3′-phosphohydrolase (CNP), myelin basic protein

INTRODUCTION

Myelination of the rodent central nervous system (CNS) is primarily a postnatal process (Morell et al., 1972; Matthieu et al., 1973a) and proceeds through two partially overlapping stages. First, there is a period of oligodendroglial proliferation and development, which occurs primarily during the first two weeks of postnatal life. This is followed by a period of rapid myelin synthesis and deposition, which commences at about 11–14 days (Matthieu et al., 1973a). The rate of myelin synthesis peaks at about day 18, then declines slowly thereafter to adult levels.

Although the time course of myelination is well understood, little is known about the signals which regulate the proliferation of oligodendrocytes and their precursors, the differentiation of oligodendrocytes, the synthesis of myelin components and the assembly of mature multilamellar myelin. The addition of physiological concentrations of insulin-like growth factor-I (IGF-I) to monolayer cultures of mixed glial cells greatly increases the number of oligodendrocytes that develop in these cultures (McMorris et al., 1986) by stimulating precursor

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cells (Raff et al., 1983; Behar et al., 1988) to proliferate and to differentiate into oligodendrocytes (McMorris and Dubois-Dalcq, 1988; McMorris et al., 1990; McMorris, unpublished data). Supraphysiological concentrations of insulin, or physiological concentrations of IGF-II, both of which activate type I IGF receptors (Rechler and Nissley, 1985) have similar effects on oligodendrocyte development (McMorris, 1983; McMorris et al., 1986, 1990; McMorris and Furlanetto, 1989). These data show that IGF-I and -II stimulate oligodendrocyte development but, since the experiments were carried out in monolayer cultures that contain few or no neurons and therefore produce little or no myelin, they do not address the question of whether IGFs affect the synthesis and accumulation of myelin.

To examine the effects of IGF-I on the synthesis and accumulation of myelin, we used aggregate cultures (Honegger and Richelson, 1976) established from 16-day fetal rat brain. Neurons, oligodendrocytes, and astrocytes develop in these cultures, and myelination occurs (Matthieu et al., 1978; Trapp et al., 1979, 1981, 1982). Several previous studies suggest that IGF-I might promote myelination in the aggregate culture system. Lenoir and Honegger (1983) showed that the specific activity of 2′-3′-cyclic nucleotide 3′-phosphohydrolase (CNP), a marker for both oligodendrocytes and myelin, is increased by IGF-I, but oligodendrocyte numbers and myelin content of the cultures were not determined. Almazan et al. (1985b) found that growth hormone elevated the amount of myelin basic protein in aggregate cultures. Because most, if not all, of the growth-related effects of growth hormone are mediated by IGF-I (Daughaday and Rotwein, 1989), this observation suggests that IGFs promote myelination. However, oligodendrocyte number and myelin content were not measured.

In the present study, we maintained aggregate cultures in the presence or absence of IGF-I or of insulin at concentrations sufficient to activate IGF receptors. We found that these treatments increase the number of oligodendrocytes that develop and the amount of myelin that accumulates in the cultures. However, neither the amount of myelin synthesized per oligodendrocyte nor the synthesis rate of myelin proteins by individual oligodendrocytes appears to be increased by IGF-I or insulin under these culture conditions.

MATERIALS AND METHODS

Aggregate Cell Cultures

Cell cultures were prepared essentially as described by Honegger and Richelson (1976). Briefly, whole brains were removed from 16-day fetal rats, strain LEC (Preston and McMorris, 1983) and were mechanically dissociated into a single cell suspension by sieving through nylon mesh of 210-μm and then 130-μm pore size. The cells were centrifuged and then resuspended in the aggregate culture medium described by Almazan et al. (1985a), which consists of Dulbecco-modified Eagle's medium (high-glucose formula, no pyruvate; GIBCO, Grand Island, NY) supplemented with vitamins, L-carnitine, triiodothyronine, hydrocortisone, α-tocopherol, lipoic acid, retinol, and trace elements as described (Almazan et al., 1985a), and further supplemented with glucose to 6 g/L total concentration, antibiotics, and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT). Micro-Fernbach flasks (25 ml, Reliance Glass Works, Inc., Bensenville, IL) containing 3.5–4.0 ml of cell suspension were placed on a horizontal rotating shaker set at 75–80 rpm. On day 2, each culture was split into two new flasks and the medium volume was adjusted to 4.0 ml per flask. Where indicated, cultures were supplemented with 100 ng/ml recombinant human IGF-I (Amgen, Thousand Oaks, CA or IMCERA., Terra Haute, IN) or 5,000 ng/ml bovine insulin (Sigma Chemical Co., St. Louis, MO). Cultures were given an 87.5% medium change every 2 days.

Myelin Isolation

Myelin was isolated as described by Norton and Poduslo (1973) with the following modification. After the first water shock and subsequent centrifugation, the pellet was resuspended in 0.85 M sucrose, overlaid with 0.32 M sucrose, and recentrifuged at 82,500*g*. As a result, myelin floats up to the interface, and fragments of denser membrane separate without sedimenting through the myelin layer and contaminating it. After the last water wash, the myelin was lyophilized and stored at −70°C for later analysis. Lyophilized myelin was resuspended in 1% sodium dodecyl sulfate (SDS) prior to biochemical analysis. Myelin yield was determined by protein assay.

Biochemical Analysis

CNP activity was assayed from aliquots of culture homogenates by the method of Prohaska et al. (1973), using toluene–isobutanol (1:1) instead of benzene–isobutanol (1:1), to extract the colored phosphomolybdic acid complex. Absorbance was measured at 410 nm. Protein was assayed by the bicinchoninic acid (BCA) modification (Smith et al., 1985) of the method of Lowry et al. (1951), using reagents purchased from Pierce Chemical Company (Rockford, IL). Bovine serum albumin (BSA) (Sigma) was used as the standard. Controls showed that the assay was not affected by the SDS present in the solubilized myelin samples.

Protein Radiolabeling in Culture

Cultures were washed three times and refed with 3.5 ml culture medium containing 1/10th the normal concentration of methionine. After 18 hr [^{35}S]methionine (Amersham, Arlington Heights, IL) was added to a concentration of 40–50 μCi/ml (2–2.5 Ci/mmol); cultures were harvested 4 hr later for myelin isolation.

Measurement of [^{35}S]Methionine Incorporation

Protein was precipitated from homogenates and myelin samples with trichloroacetic acid (TCA) essentially as described by Mans and Novelli (1961). Radioactivity was determined by liquid scintillation counting and normalized to total culture protein. To determine the amount of radiolabeled methionine which could bind nonspecifically to myelin, 9 μCi of [^{35}S]methionine was added to homogenate from a 31-day-old rat brain, and myelin was then isolated. No radioactivity, over the background levels, was detected in the myelin.

Immunohistochemistry

Aggregates were washed three times with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 11.5 mM Na_2HPO_4, 14.7 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ pH 7.3) and then fixed in 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.5, for 1 hr. Aggregates were then washed three times with PBS, equilibrated sequentially in 20%, 60%, and 80% glycerol in 0.1 M phosphate buffer, pH 7.5 (v/v) for 15 min each and then in 96% glycerol for 60 min. Aggregates were stored at −70°C until used. For sectioning, aggregates were warmed to room temperature, equilibrated to 2% (wt/vol) sucrose in 0.1 M sodium phosphate, pH 7.5, and stored overnight at 4°C in 10% (w/v) sucrose. Aggregates were then frozen in OCT embedding medium (Miles Labs, Naperville, IL) at −20°C for 1 hr and sectioned at 8 μm on a cryostat. Sections were mounted on slides coated with subbing solution [0.5% (w/v) gelatin, 0.05% (w/v) $CK(SO_4)2$].

Antisera against purified bovine brain CNP and guinea pig spinal cord MBP were prepared in rabbits as described (Raible and McMorris, 1989; McMorris et al., 1981). The anti-CNP antiserum was absorbed against immobilized MBP to remove minor anti-MBP reactivity. Both antisera showed strong reactivity against their respective antigens and little or no reactivity against other proteins, in immunoblots of rat CNS myelin or whole rat brain homogenate. Nonimmune rabbit serum was used as control in all experiments.

Cells were immunostained by the avidin–biotin complex (ABC) immunoperoxidase procedure, using reagents purchased from Vector Laboratories (Burlingame, CA). Briefly, sections or monolayer cultures were incubated at room temperature for 1 hr each in antiserum diluted 1:500 in Tris buffer [TB: 0.05 M Tris (Sigma), 0.4 M NaCl, pH 7.5] containing 5% goat serum, followed by biotinylated goat antirabbit IgG diluted 1:200 in TB containing 5% goat serum, and then avidin-biotinylated horseradish peroxidase (HRP) complex, with two 15-min rinses in TB between each step. Color was developed in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma), 0.01% hydrogen peroxide in 0.05 M Tris, pH 7.2 for 10 min. Slides were then counterstained with Mayer's hematoxylin, dehydrated, and mounted with Gurr's neutral mounting medium (Atomergic Chemicals Corp., Farmingdale, NY).

Determination of Cell Number

Stained slides were examined at 250–400× magnification by bright-field and differential interference contrast microscopy. A cell was considered positive for a particular antigen if it stained dark brown and contained a blue nucleus. The total number of oligodendrocytes in each section is expressed at the number of CNP-positive or MBP-positive cells per mm^2. Data were corrected by the method of Abercrombie [1946] for the probability of counting the same cell twice in sequential sections. Means and SEM were calculated from 3–9 sections from a given age and treatment.

RESULTS

Development of Oligodendrocytes in Aggregate Cultures

Aggregate cultures established from 16-day fetal rat brain were analyzed after 13, 20, or 27 days in vitro (corresponding to postnatal days 8, 15, and 22). Sections were immunostained with antisera specific for CNP or MBP and counterstained with hematoxylin. A cell was scored as an oligodendrocyte if it had a dark brown immunostained cytoplasm clearly surrounding a blue hematoxylin-stained nucleus. Thus, cells in which the nucleus lay outside the plane of the section were intentionally excluded; but oligodendrocytes with dark processes and a lightly immunostained perinuclear cytoplasm or oligodendrocytes stained so darkly that the nucleus was obscured would have inadvertently been omitted.

Figure 1 shows the results of representative experiments for CNP and MBP immunostaining. Oligodendrocytes were present in small numbers at the earliest time observed. Their numbers increased with subsequent time in culture, reminiscent of the normal time course of oligodendrocyte development in vivo and in agreement with previous observations in aggregate cultures (Mathieu et al., 1978; Trapp et al., 1979, 1982).

When 100 ng/ml IGF-I was present in the culture medium, the number of oligodendrocytes was increased

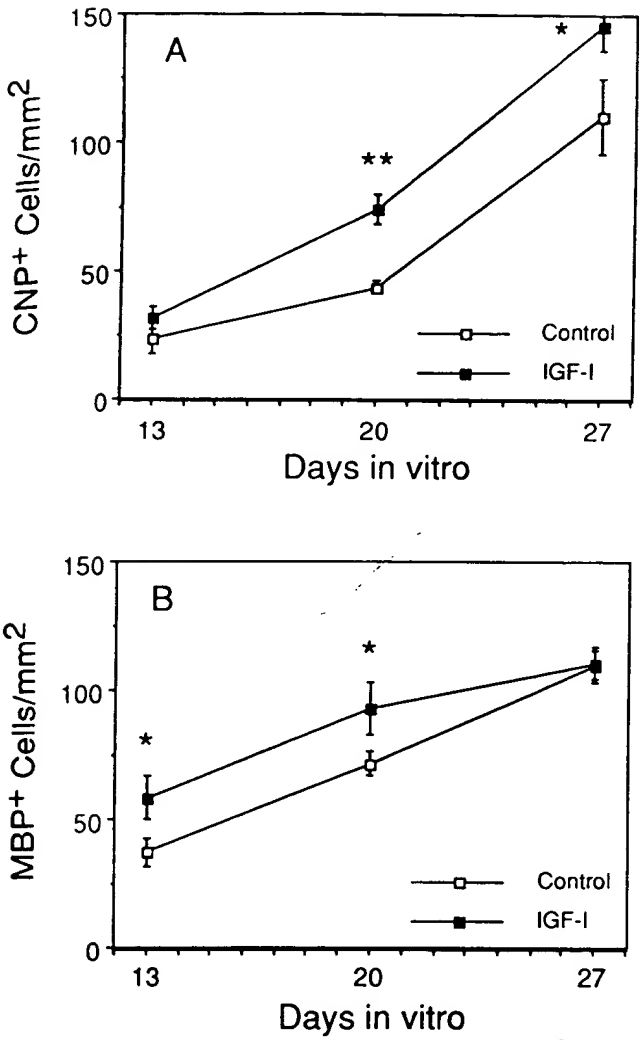

Fig. 1. IGF-I increased the number of oligodendrocytes that develop in aggregate cultures. Cultures were given IGF-I at 100 ng/ml starting on day 2 in vitro, and the number of oligodendrocytes per mm^2 was determined in immunostained frozen sections at the times shown. A representative experiment is shown for CNP-positive (**A**) and for MBP-positive oligodendrocytes (**B**). Symbols show the mean ± SEM for 3–9 tissue sections from triplicate cultures for each treatment. The absolute number of oligodendrocytes varied from one experiment to the next, but the patterns for all experiments were very similar to those shown. For statistical analysis, each data point was expressed as the percentage of the mean of the controls for that day within the same experiment, and the pooled date from four experiments for CNP or from three experiments for MBP were analyzed by the Mann–Whitney U test. *significantly different from control at $P<0.05$; **statistically different from control at $p<0.001$.

over control values (Fig. 1A,B). The pattern was similar for CNP-positive and MBP-positive cells, except at day 27, when the number of MBP-positive cells was unaffected by the IGF-I treatment. This was an unexpected but consistent finding. The result may reflect an intrinsic property of the effect of IGF-I on the expression of MBP, which appears later in oligodendrocyte development than CNP. Alternatively, the result may be an artifact due to the fact that, in more mature oligodendrocytes, MBP is localized primarily in the myelin sheaths and oligodendroglial processes rather than in the cell body (Sternberger et al., 1985). Thus, the most mature oligodendrocytes may not have been detected in the MBP-immunostained preparations, and if the oligodendrocytes are more mature in the IGF-I-treated cultures than in the controls, their numbers would be underestimated by this technique.

CNP Activity in Aggregate Cultures

Cultures were assayed for the activity of CNP, which is present at very high activity in CNS myelin and has been used as an index of myelin content in vivo (Sims and Carnegie, 1978; Sprinkle et al., 1978). CNP is also very abundant in oligodendroglial cell bodies and in premyelinating oligodendrocytes, and activity increases as oligodendrocytes mature (Matthieu et al., 1973b; Waehneldt, 1975; Sims and Carnegie, 1978; McMorris, 1983; Raible and McMorris, 1989); thus, CNP activity is a reflection of oligodendrocyte numbers, oligodendrocyte maturity and myelin content. Figure 2 shows the CNP content of cultures in a representative experiment. The CNP activity of the cultures was high at the earliest time examined, as would be expected since oligodendrocytes were already present at that time (cf. Fig. 1). CNP activity increased three- to fourfold from day 13 to day 27 of culture, in agreement with the observed increase in oligodendrocyte numbers, and consistent with an increase in oligodendrocyte maturity and the presence of myelin. In cultures grown in the presence of 100 ng/ml IGF-I, CNP activity was increased by as much as 2.5-fold over controls (Fig. 2). In individual experiments, the increase in CNP activity in response to IGF-I was occasionally as great as fivefold (data not shown).

Myelin Content of Aggregate Cultures

Because the CNP data did not allow us to distinguish between changes in oligodendrocyte number and/or maturity and changes in myelin content in response to IGF-I, we measured the myelin content of the cultures directly. Myelin was isolated from homogenates of aggregate cultures by the method of Norton and Poduslo (1973), with an additional step to further reduce contamination by nonmyelin membranes (see under Methods). Figure 3 shows the results of a representative experiment. A small amount of myelin could be isolated from control cultures at day 13 or day 20 after explantation, and the amount increased by day 27. Addition of 100

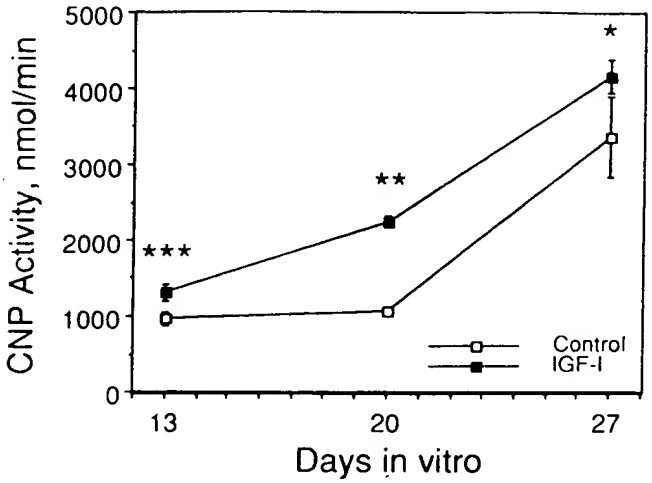

Fig. 2. IGF-I increased the CNP activity expressed in developing aggregate cultures. Cultures were grown as described under Methods and in the legend to Figure 1, and the total CNP activity per culture was determined at the times shown. The data represent the means to duplicate CNP assays on triplicate cultures for each condition, ±SEM. Data are shown from one experiment, which is representative of five experiments with IGF-I and from two experiments in which 5,000 ng/ml insulin was used instead of 100 ng/ml IGF-I. The absolute values for CNP activity varied from experiment to experiment, but the patterns for all experiments were similar to those shown. For statistical analysis data were normalized as described in the legend to Figure 1, and the pooled data from five experiments for IGF-I were analyzed by the Mann–Whitney U test. *significantly different from control at $P<0.01$, **$P<0.002$, ***$P<0.001$.

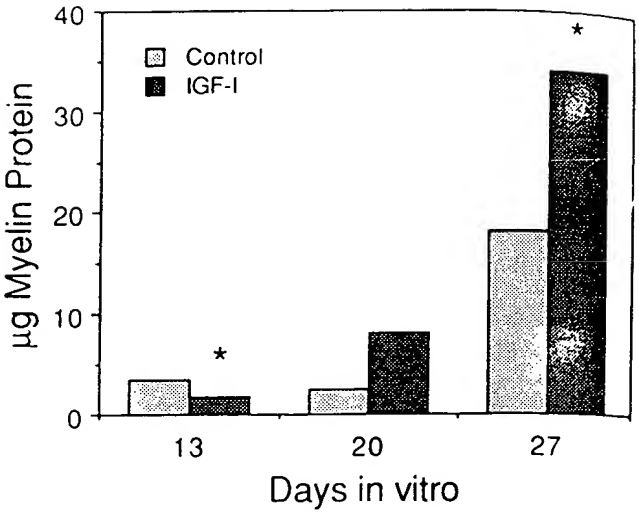

Fig. 3. IGF-I increased the total amount of myelin in aggregate cultures. Cultures were grown and myelin was isolated and quantitated as described under Methods. The mean values are shown from one experiment representative of three experiments, which gave similar results. For statistical analysis, data from the three experiments were normalized as described in the legend to Figure 1, pooled, and analyzed by the Mann–Whitney U test. *significantly different from control at $P<0.05$.

ng/ml IGF-I to the culture medium resulted in approximately a doubling in the myelin yield at day 20 or day 27. At day 13, however, the myelin yield was actually reduced by 30–40% in the IGF-I-treated cultures. This was an unexpected but consistent finding. The increase in myelin yield in response to IGF-I at day 20 and day 27 (Fig. 3) was of the same order of magnitude as the increase in oligodendrocyte numbers at the same time (Fig. 1).

Effect of High Concentrations of Insulin

At supraphysiological concentrations (e.g., 5,000 ng/ml), insulin has similar effects on oligodendrocyte development as physiological concentrations of IGF-I or IGF-II (100 or 230 ng/ml, respectively) (McMorris et al., 1986, 1990; McMorris and Furlanetto, 1989). At these high concentrations, insulin binds to type I IGF receptors and acts as an analogue of IGF-I and IGF-II (Massague and Czech, 1982; Perdue, 1984; Rechler and Nissley, 1985; Ewton et al., 1987). The type I IGF receptor is the receptor that mediates the action of IGF-I, IGF-II and insulin on the development of oligodendrocytes (McMorris and Furlametto, 1989) as well as in other systems (Ewton et al., 1987; Kiess et al., 1987). When cultures were incubated with 5,000 ng/ml insulin rather than 100 ng/ml IGF-I starting on day 2 of culture and harvested on day 20, we observed an increase in oligodendrocyte number, CNP activity and myelin content very similar to that observed with IGF-I (Fig. 4; cf. Figs. 1–3).

Synthesis Rate of Myelin Proteins in Aggregate Cultures

In principle, the increase in myelin accumulation observed in the IGF-I- or insulin-treated cultures might be due to an increase in rate of myelin synthesis per oligodendrocyte, or it might simply be a consequence of the fact that the hormone-treated cultures contain more oligodendrocytes. The results of the experiments above are consistent with the interpretation that the increased myelin yield is largely due to the increase in the numbers of oligodendrocytes that develop in cultures in the presence of IGF-I or insulin. In order to determine whether IGF-I or insulin might also increase the rate at which individual oligodendrocytes synthesize and accumulate myelin, we measured the synthesis rate of myelin proteins in aggregate cultures in the presence or absence of the hormones, under conditions where the number of oligodendrocytes was unaffected by the hormone treat-

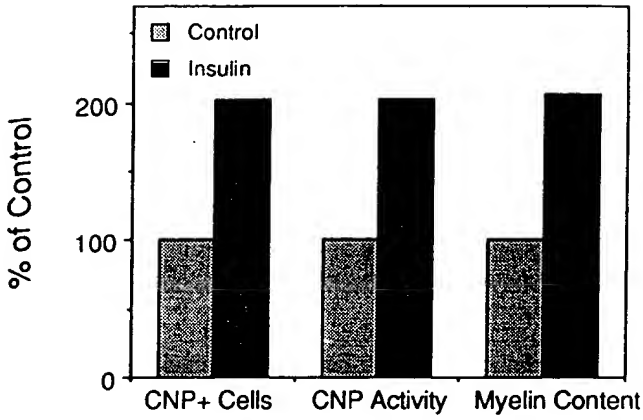

Fig. 4. 5,000 ng/ml insulin mimics the effects of 100 ng/ml IGF-I on oligodendrocyte development and myelination in aggregate cultures. Cultures were maintained and analyzed as described in the legends to Figures 1–3, except that experimental cultures were treated with 5,000 ng/ml insulin, rather than 100 ng/ml IGF-I, starting on day 2 after explantation. On day 20, cultures were harvested and the number of CNP-positive cells per mm^2, the total CNP activity per culture, and the total amount of myelin per culture were determined for control and insulin-treated cultures. The data shown are the means of triplicate cultures for each condition from a representative experiment, expressed as the percentage of control.

ment. These experiments were carried out using insulin as an analogue of IGF-I.

When insulin treatment of aggregate cultures was started at day 20 after explanation, rather than day 2, there was no significant difference in the number of CNP-positive or MBP-positive oligodendrocytes in the treated cultures, as compared with controls, on days 22–28 (Fig. 5A,B). Thus, any difference in the amount of myelin present in the cultures or in the rate of myelin protein synthesis could be interpreted as a difference in the amount of myelin produced per oligodendrocyte or in the rate of myelin synthesis per oligodendrocyte, in the presence of insulin.

Myelin was isolated from control cultures and cultures treated with insulin from day 20, and the total amount of myelin was quantitated by protein determination. Figure 6 shows that the total amount of myelin in the cultures increased from day 20 to day 26. However, there was no significant difference in myelin content between the control and insulin-treated cultures during this time period. Together with the results shown in Figure 5, this indicates that treatment with insulin during the day-20–day-26 period does not affect the amount of myelin produced per oligodendrocyte in aggregate cultures. However, we cannot rule out the possibility that a difference would have been observed after a longer period of insulin treatment. Likewise, a difference in accumu-

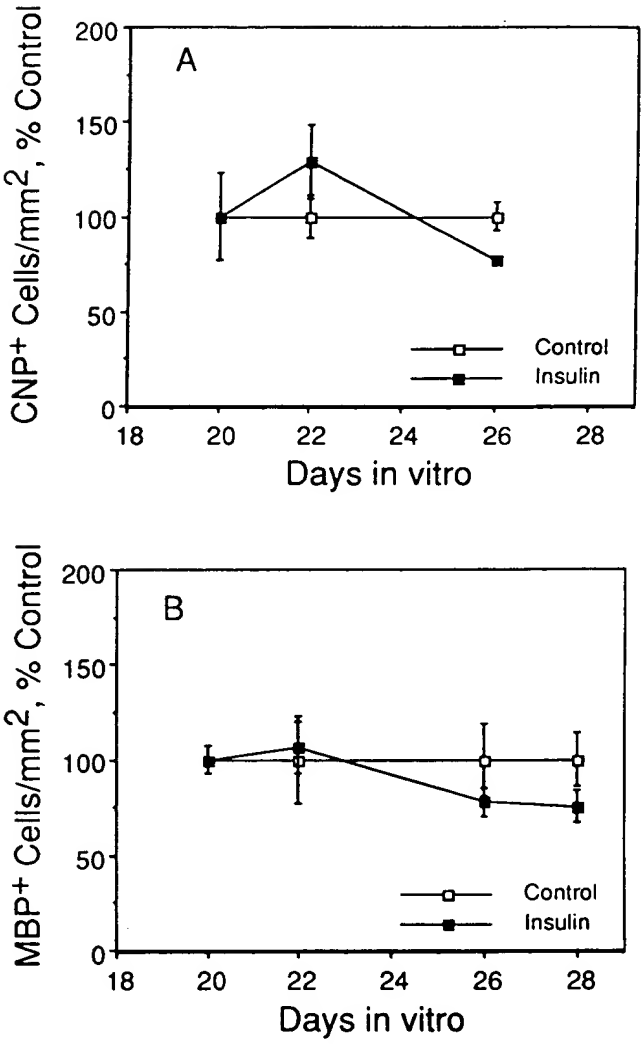

Fig. 5. Insulin treatment starting at day 20 did not alter the number of oligodendrocytes in aggregate cultures. The number of CNP-positive cells (A) or MBP-positive cells (B) per mm^2 was determined at the time points indicated in control cultures and in cultures treated with 5,000 ng/ml insulin. Each point represents the mean of 3–5 sections ±SEM. No significant difference between insulin and control at any time point as determined by Student's *t*-test.

lation of less than approximately 25% would have been undetectable by these methods.

To measure the synthesis rate of myelin proteins directly, control and insulin-treated cultures were incubated with [^{35}S]methionine for 4 hr; the cultures were then homogenized and myelin was isolated. The amount of radioactivity incorporated into myelin protein was determined for each condition, and compared with the amount of radioactivity incorporated into total protein in the same culture. Figure 7 shows that approximately 7% of the total protein synthesis during the 4-hr labeling period represented synthesis of myelin protein. This was

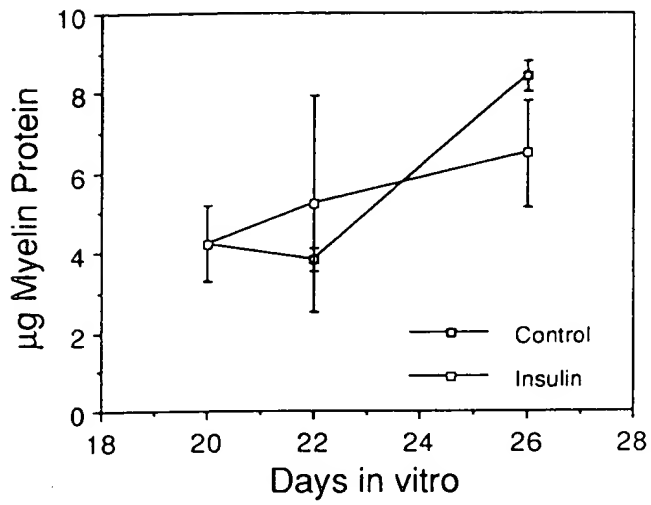

Fig. 6. Insulin treatment starting at day 20 did not alter the myelin yield from aggregate cultures. The myelin yield was determined at the time points indicated in control cultures and in cultures treated with 5,000 ng/ml insulin as described under Materials and Methods. Each point represents the mean of three cultures ±SEM. No significant difference between insulin and control at any time point as determined by Student's *t*-test.

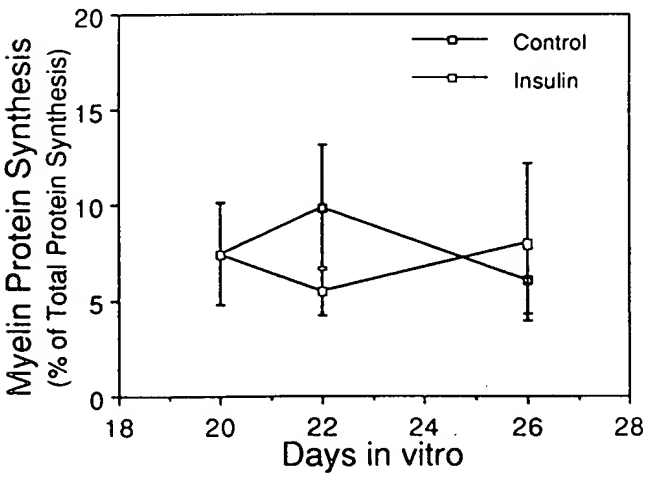

Fig. 7. Insulin treatment starting at day 20 did not alter the synthesis rate of myelin protein in aggregate cultures. Incorporation of [^{35}S]methionine into myelin protein and into total protein was determined at the time points indicated in control cultures and in cultures treated with 5,000 ng/ml insulin as described under Materials and Methods. Each point represents the mean of three cultures ±SEM. No significant difference between insulin and control at any time point as determined by Student's *t*-test.

true at all times examined (days 20, 22 and 26 after explanation), and was not affected by treatment with insulin. Thus, under this treatment paradigm in which similar numbers of oligodendrocytes are present in the treated and control cultures, insulin had no significant effect on the synthesis rate of myelin proteins. Thus, we conclude that the increase in myelin content of cultures treated with IGF-I or insulin starting on day 2 of culture (Figs. 3, 4) is primarily the consequence of the larger numbers of oligodendrocytes present in those cultures.

DISCUSSION

The data presented here show that IGF-I stimulates a significant increase in the number of oligodendrocytes that develop in aggregate cultures. In these cultures, 100 ng/ml IGF-I stimulated a 35–80% increase in the number of oligodendrocytes compared with control cultures. Thus, our observations reported here in three-dimensional aggregate cultures of neurons and glia agree with our previous findings from surface cultures of glia, which showed that physiological concentrations of IGF-I or IGF-II, or supraphysiological concentrations of insulin that can interact with type I IGF receptors, increase the number of oligodendrocytes that develop in vitro (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988; McMorris and Furlanetto, 1989).

The increase in oligodendrocyte numbers we observed was probably the result of the action of IGF-I on oligodendrocyte precursors and young oligodendrocytes. Oligodendrocytes cannot be detected in 4-day-old aggregate cultures (Trapp et al., 1979), thus, the oligodendrocytes present at later times must arise from precursors. In surface cultures, IGF-I promotes the proliferation of oligodendrocyte precursors and young oligodendrocytes, and also induces immature glial cell precursors to develop into oligodendrocytes (McMorris and Dubois-Dalcq, 1988; McMorris et al., 1990). Thus, it is likely that similar processes are responsible for the increase in oligodendrocyte numbers in aggregate cultures treated with IGF-I. However, we cannot rule out the possibility that IGF-I also acts, in part, as a survival factor for oligodendrocytes.

Since type I IGF receptors are present on oligodendrocytes and oligodendrocyte precursors (McMorris et al., 1986), it is likely that the observed increase in oligodendrocyte development is due to direct interaction of IGF-I with these cells. However, we cannot rule out the possibility that secondary effects, resulting from interaction of IGF-I with other cell types in the cultures, affect the development of oligodendrocytes in IGF-I-treated cultures.

In addition to increasing the number of oligodendrocytes that developed in the cultures, IGF-I increased the amount of myelin which accumulated. By days 20–27 in vitro (corresponding to postnatal day 15–22), the amount of myelin that could be isolated from IGF-I-

treated cultures was 35–90% greater than that of controls. Thus, in addition to its effects on the number of oligodendrocytes that develop, as previously observed in surface cultures, IGF-I also significantly enhances the accumulation of myelin.

In our cultures, the increase in myelin yield could be fully accounted for by the increase in the numbers of oligodendrocytes in the IGF-I-treated cultures, and could not be accounted for by an increased rate of myelin synthesis or an increase in the amount of myelin produced per oligodendrocyte in the presence of IGF-I. It is possible that a longer period of development in the presence of IGF-I would result in an increase in the amount of myelin per oligodendrocyte as well as an increase in oligodendrocyte numbers. In transgenic mice that overexpress IGF-I in the CNS, we have observed an increase in the amount of myelin produced per oligodendrocyte in vivo which, together with a small increase in the number of oligodendrocytes, resulted in a doubling in the total amount of myelin in the CNS by postnatal day 56 (Carson, 1990; Carson et al., 1988a,b, 1989).

The earliest time point at which we quantitated myelin content in the cultures, day 13, corresponds to postnatal day 8, a time at which very little myelin has developed in brain in vivo. At this time point, we consistently observed a significant (approximately 30%) decrease in myelin content of the IGF-I-treated cultures as compared with control. Although this finding was unexpected, it is consistent with the notion that prolonged proliferation of oligodendrocytes or their precursors in response to IGF-I might have temporarily delayed the onset of myelin synthesis.

Both IGF-I and IGF-II are present in brain prenatally and postnatally (D'Ercole et al., 1980, 1984; Binoux et al., 1981; Daughaday and Rotwein, 1989; Hepler and Lund, 1990) at concentrations that affect oligodendrocyte development in vitro (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988; McMorris and Furlanetto, 1989).

In addition, mRNA for both IGF-I and IGF-II are present in brain (Brown et al., 1986; Matthews et al., 1986; Lund et al., 1986; Rotwein et al., 1988), although the relative contribution of locally synthesized and blood-borne IGFs in brain development remain undetermined. Previous studies in surface cultures have shown that IGF-I promotes the development of oligodendrocytes (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988; McMorris and Furlanetto, 1989), but it could not be determined from those studies whether this would ultimately result in any effect on myelination. In the present study, we have used a culture system in which neurons are abundant and significant quantities of myelin are produced, and have shown that IGF-I does, in fact, increase the myelin content of the cultures.

ACKNOWLEDGMENTS

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Insulin-like Growth Factor I Increases Brain Growth and Central Nervous System Myelination in Transgenic Mice

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Summary

Insulin-like growth factor I (IGF-I) is a potent regulator of oligodendrocyte development and myelination in vitro, but its effect on myelination in vivo has never been tested directly. Therefore, we examined brain growth and myelination in a transgenic mouse line that overexpresses IGF-I. By postnatal day 55, when brain growth and myelination are essentially complete in normal mice, the brains of transgenic mice were 55% larger than those of controls owing to an increase in cell size and apparently in cell number. Most or all brain structures appeared to be affected. At the same time, total myelin content of the transgenic mice was 130% greater than that of controls. Oligodendrocyte number as a percentage of total cell number was not increased in the transgenic mouse brains; the increase in myelin content was primarily the result of an increase in myelin production per oligodendrocyte. These findings indicate that IGF-I is a potent inducer of brain growth and myelination in vivo.

Introduction

Myelin in the central nervous system (CNS) is produced by oligodendrocytes, one of the most abundant cell types in the CNS (Norton, 1984), and is essential for normal CNS function. However, despite extensive studies of oligodendrocyte development and myelination, relatively little is known about the regulation of these processes. Nevertheless, several factors have been identified that affect the development of oligodendrocytes in in vitro model systems. Among the most potent regulators of oligodendrocyte development are the insulin-like growth factors, IGF-I and IGF-II (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988; Mozell and McMorris, 1991).

Very few, if any, oligodendrocytes are present in rat cerebrum on postnatal day 1, but if the cells are dissociated and cultured in vitro in medium containing 10% fetal bovine serum, oligodendrocytes develop during the ensuing few weeks on a time course similar to that observed in vivo (Abney et al., 1981; McMorris, 1983). Addition of physiological concentrations of IGF-I or IGF-II, or of insulin at high concentrations that cross-react with type I IGF receptors, increases the number of oligodendrocytes that develop in the cultures up to 6-fold, while having very little effect on the numbers of other cell types (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988; Mozell and McMorris, 1991). If serum is omitted, very few oligodendrocytes develop in these cultures; however, IGF-I, IGF-II, or a high concentration of insulin increases the number of oligodendrocytes that develop up to 60-fold over baseline (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988). IGFs increase oligodendrocyte numbers by acting as mitogens and by inducing immature glial precursors to develop into oligodendrocytes (McMorris and Dubois-Dalcq, 1988; McMorris et al., 1990). The effects of all three insulin family peptides on oligodendrocyte development are mediated by type I IGF receptors, which are present on oligodendrocytes and their precursors (McMorris et al., 1986, 1990; F. A. McMorris and R. W. Furlanetto, unpublished data). In CNS aggregate cultures containing neurons as well as glial cells, IGF-I increases both the oligodendrocyte number and the amount of myelin that is produced (Mozell and McMorris, 1991). It has recently been reported that IGF-I is also a survival factor for oligodendrocytes and oligodendrocyte precursors (Barres et al., 1992).

Evidence for a role for IGFs in oligodendrocyte development and myelination in vivo is more indirect. Rats and mice that are growth hormone deficient, as the result of either administration of antibody against growth hormone or the Snell *dwarf* (*dw*) or *little* (*lt*) mutations, are hypomyelinated in the CNS, and the hypomyelination is associated with a deficiency in oligodendrocyte numbers (Pelton et al., 1977; Noguchi et al., 1982a, 1982b, 1985; Sugisaki et al., 1985; King et al., 1988). Because most of the effects of growth hormone are mediated by IGF-I (Froesch et al., 1985; Mathews et al., 1986; Fagin et al., 1989), these observations implicate IGF-I as a regulator of CNS myelination (McMorris et al., 1986). Severe undernutrition lowers serum IGF-I without lowering growth hormone levels (Grant et al., 1973; Hintz et al., 1978; Phillips et al., 1978; Phillips and Vassilopoulou-Sellin, 1979) and results in irreversible CNS hypomyelination (Krigman and Hogan, 1976; Delaney et al., 1981; Wiggins, 1982; Egwim et al., 1986), further implicating IGF-I in myelination (McMorris et al., 1986). IGF-I and IGF-II and their mRNAs are present in brain prenatally and in the early postnatal period when oligodendrocyte development and myelination are occurring (D'Ercole et al., 1984; Brown et al., 1986; Lund et al., 1986; Rotwein et al., 1988). Taken together, these observations suggest that IGFs are required for normal CNS oligodendrocyte development and myelination in vivo. However, the

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effect of IGF-I on CNS myelination in vivo has never been directly tested.

To examine whether IGF-I regulates myelination in vivo, we examined brain growth and myelination in a transgenic mouse line that carries a human IGF-IA cDNA sequence under the transcriptional control of a mouse metallothionein promoter (Mathews et al., 1988). As detected by radioimmunoassay, serum IGF-I levels in the transgenic animals were elevated 1.5- to 1.6-fold over those of the controls, and brain IGF-I levels were elevated 1.5- to 2-fold (Mathews et al., 1988). We found that, whereas there was little difference in body size, the transgenic mice had brains that were 50%–70% larger and contained 125%–130% more myelin than those of their normal, nontransgenic littermates.

Results

Brain Size Is Increased in IGF-I Transgenic Mice

The IGF-I transgene was maintained in a hemizygous condition in the IGF-I transgenic mouse line. Therefore, each litter consisted of approximately equal numbers of transgenic and nontransgenic mice. Transgenic and nontransgenic siblings were not readily distinguishable in size (Figure 1A), although transgenic mice were up to 25% heavier than their nontransgenic siblings by 2 months of age (Table 1). Additionally, the foreheads of the transgenic mice were slightly but consistently larger and more domed than those of their nontransgenic littermates.

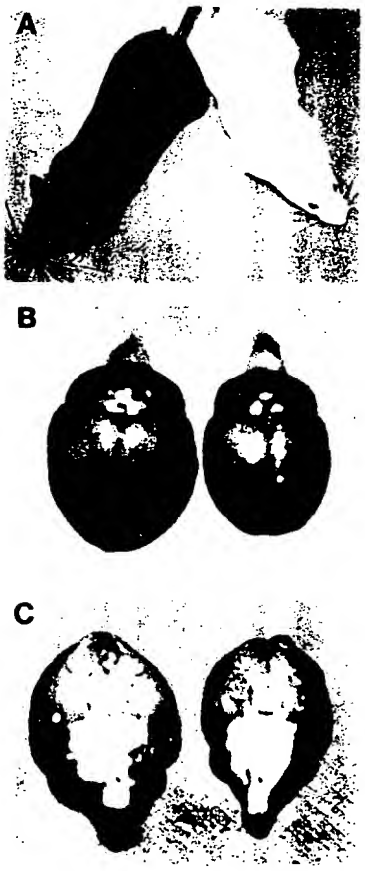


Figure 1. Body Size and Brain Size of IGF-I Transgenic and Control Mice

(A) Two female littermates at 56 days of age. The mouse on the left carries the IGF-I transgene, whereas the mouse on the right does not. (Coat color does not cosegregate with the transgene.) (B) Dorsal and (C) ventral views of the brains from the mice in (A) at day 56. The brain on the left is from the transgenic mouse

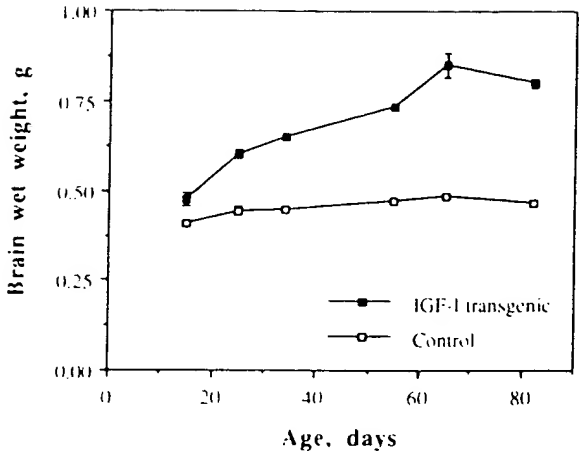


Figure 2. Brain Weight of IGF-I Transgenic and Control Mice

Brains were rapidly removed from mice at the ages shown and weighed. Each data point represents a minimum of two experiments and eight mice per condition except for day 65, which represents one experiment and three mice per condition. Error bars represent SEM; when not shown, the error bars are smaller than the symbols.

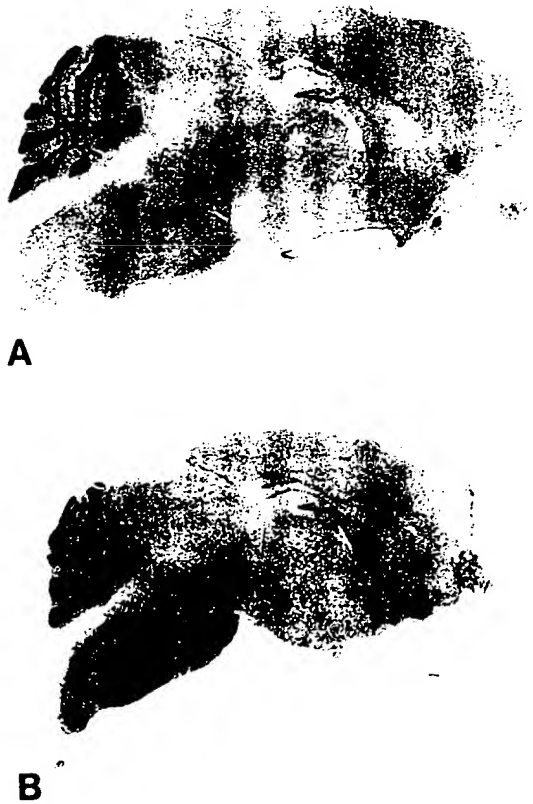
In contrast with the small differences in body size, the brain sizes of the control and the transgenic mice were strikingly different (Figures 1B and 1C; Table 1). The difference in brain weight was apparent by day 15, which was the earliest time point examined (Figure 2). At this time, brains of the transgenic mice were 15%–20% heavier than those of their control siblings. Between days 15 and 55, brain weight in the control mice increased by 25%, whereas brain weight in the transgenic mice increased by 90%. Consequently, brains from the transgenic mice were 55% heavier than brains from their nontransgenic siblings by day 55 and 70% heavier by day 82 (Figure 2).

Gross examination of brains of the transgenic mice revealed a uniform enlargement (Figures 1B and 1C), and in histological sections, all brain structures appeared to be enlarged to a similar extent with no obvious abnormalities (Figure 3). Weight determinations for major brain regions (cerebrum, midbrain, cerebellum, diencephalon, and brain stem) also revealed increases in all regions (Figure 4), with relatively myelin-rich regions showing somewhat greater enlargement than brain as a whole (transgenic/control = 1.96 and 1.93 for midbrain and brain stem, respectively, as compared with 1.81 for whole brain) and less heavily myelinated regions showing somewhat less enlargement

Table 1. Comparison of Body Weight, Brain Weight, and Total Brain Content of Protein, Myelin, and DNA in IGF-I Transgenic Mice and Control Littermates

Measurement	Control, Mean ± SEM	Transgenic, Mean ± SEM	Transgenic, % Greater than control	p
Day 33				
Body weight, g	22.18 ± 1.08	23.60 ± 0.59	6	NS
Brain weight, g	0.462 ± 0.002	0.657 ± 0.007	42	<0.0001
Brain protein, mg	48.24 ± 0.43	65.26 ± 0.62	35	<0.0001
Brain DNA, mg	1.063 ± 0.061	1.264 ± 0.077	19	NS
Brain myelin, mg	6.597 ± 0.388	12.247 ± 0.672	86	<0.002
Myelin/body wt.	0.297 ± 0.011	0.519 ± 0.027	75	<0.002
Myelin/brain wt.	14.29 ± 0.82	18.63 ± 0.83	30	0.02
Myelin/brain protein	0.137 ± 0.007	0.188 ± 0.011	37	0.02
Myelin/brain DNA	6.21 ± 0.15	9.73 ± 0.56	57	<0.005
Day 55				
Body weight, g	25.50 ± 0.68	31.95 ± 1.37	25	<0.02
Brain weight, g	0.470 ± 0.006	0.730 ± 0.012	55	<0.0001
Brain protein, mg	52.11 ± 0.71	76.80 ± 1.59	47	<0.0001
Brain DNA, mg	1.435 ± 0.084	1.814 ± 0.234	26	NS
Brain myelin, mg	9.177 ± 0.664	21.313 ± 1.788	132	<0.005
Myelin/body wt.	0.359 ± 0.017	0.666 ± 0.040	86	0.002
Myelin/brain wt.	19.52 ± 1.36	29.15 ± 2.12	49	<0.02
Myelin/brain protein	0.176 ± 0.015	0.277 ± 0.018	57	<0.02
Myelin/brain DNA	6.41 ± 0.41	11.94 ± 1.01	86	<0.01

Transgenic and control littermate mice were killed at the ages indicated, and the brains were removed and homogenized. Aliquots of each homogenate were used for determination of protein, DNA, and myelin content as described in Experimental Procedures. Brain myelin content was normalized to body weight, brain weight, brain protein, and brain DNA content for each mouse individually. Data for control and transgenic mice were compared using Student's t test; NS indicates not significant at $p > 0.10$. The data are from two representative litters with three control and three transgenic mice each.



(1.77 and 1.70 for cerebrum and cerebellum, respectively) (Figure 4).

Total protein content of the transgenic mouse brains was increased by approximately the same proportion as brain weight (Table 1). There was no significant difference in the water content of the brains (77% ± 3% of brain weight in transgenic mice and 79% ± 1% in control mice, as measured on day 35; data not shown).

DNA content of the control and the transgenic mouse brains was determined as an indication of total cell number. To avoid any losses during DNA purification, DNA was assayed in whole tissue homogenates. Representative data are shown in Table 1. DNA content of the transgenic mouse brains was 17% greater than that of the controls at day 20 and approximately 25% greater than that of the controls by days 55–60 (Table 1; data not shown). DNA measurements were consistently greater in the transgenic mouse brains than in the controls, but the differences were not statistically significant at any time point, probably be-

Figure 3. Histological Appearance of IGF-I Transgenic and Control Mouse Brains

Brains were fixed in 4.5% paraformaldehyde by perfusion, embedded in paraffin, sectioned, and stained with hematoxylin. Midsagittal sections of brains from 60-day-old mice are shown at the same magnification. (A) transgenic mouse; (B) control mouse.

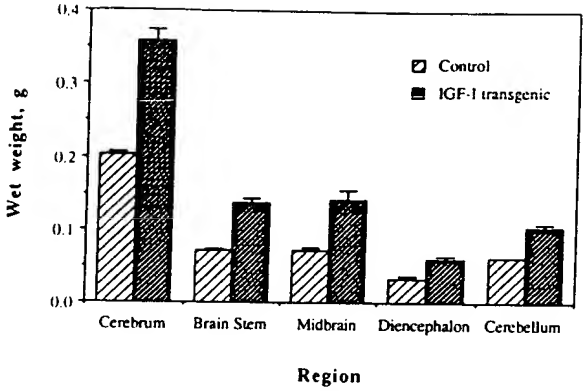


Figure 4. Regional Brain Weight in IGF-I Transgenic and Control Mice

Brains were removed from control and transgenic mice at day 56, dissected into five regions as indicated, and weighed. Each bar represents the mean of three mice ± SEM. Ratios of the weights for transgenic versus control mice were midbrain, 1.96; brain stem, 1.93; diencephalon, 1.79; cerebrum, 1.77; cerebellum, 1.70; and whole brain, 1.81.

cause of the small mean differences and mouse to mouse variations within the transgenic group. Our findings are in close agreement with those of Mathews et al. (1988), who found a nonsignificant 25% increase in brain DNA content in the same transgenic mouse line at a similar age. DNA content was less affected than brain weight and protein content: at day 33, mean DNA content was increased by approximately 20% in the transgenic mouse brains, whereas brain weight and protein content were increased by approximately 40%; and at day 55, DNA was increased by 25% and brain weight and protein content by approximately 50%. Thus, if the apparent increase in DNA content is real, approximately half of the increase in brain size of the transgenic mice could be attributed to an increase in cell number and the other half to an increase in cell size and protein content.

Transgenic Mice Have Hypermyelinated Brains

Very little myelin is present in the mouse brain at postnatal day 15, and there was no significant increase in the control (Figure 5A), which suggests that IGF-I does not accelerate the onset of myelination. However, once myelination began, myelin accumulated at a faster rate and reached a higher level in the transgenic mouse brains (Figure 5A). Myelin content per brain increased 4-fold by day 25 and nearly 11-fold by day 82 in brains of transgenic mice, as compared with increases of 3-fold and 5.6-fold at these time points in the brains of their nontransgenic siblings. As a consequence, by day 55, the total my lin content per brain was more than 2-fold greater in transgenic mice than in controls (Figure 5A; Table 1).

Because the transgenic mouse brains were larger than the controls, they would be expected to contain more myelin. In fact, myelin content was increased

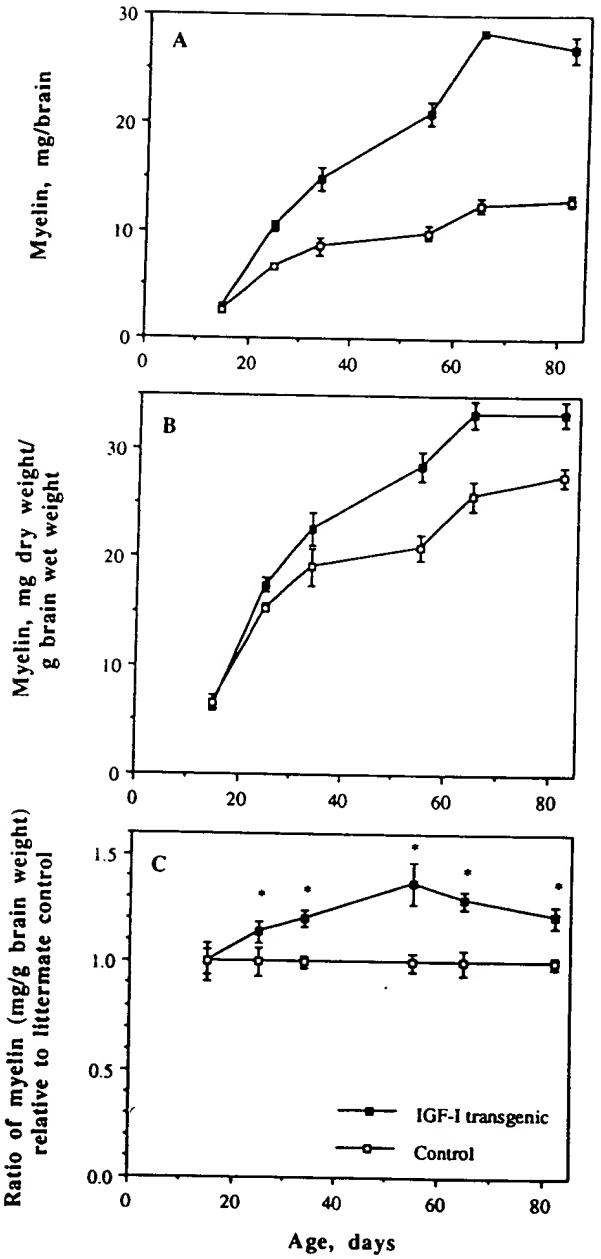


Figure 5. Myelin Content of Brains of IGF-I Transgenic and Control Mice

Brains were removed at the times indicated, and myelin was isolated, purified, and quantified as described in Experimental Procedures. Each data point represents a minimum of two experiments and eight mice per condition except for day 65, which represents one experiment and three mice per condition. Data are expressed as (A) mg of myelin (dry weight) per brain; (B) mg of myelin (dry weight) per g of brain (wet weight); (C) mg of myelin per g of brain, shown as the ratio of transgenic/control. In (A) and (B), data from several litters were pooled for each time point (except for day 65, which represents one litter). In (C), the ratio (myelin weight/brain weight) for each mouse (transgenic or nontransgenic) was divided by the mean value for the nontransgenic mice from the same litter. The data in (C) were compared by Student's t test; * indicates significantly greater than control ($p < 0.02$). Error bars represent SEM; when not shown, the error bars are smaller than the symbols.

in the transgenic mouse brains, but the increase was too large to be explained simply by the increase in brain size. This is illustrated by data from the litters represented in Table 1. At day 33, brain weight, protein content, and DNA content of the transgenic mice were 42%, 35%, and 19% greater, respectively, than those of the controls, whereas myelin content of the transgenic mice was 86% greater than that of the controls. At day 55, the increases were 55%, 47%, and 26% for brain weight, protein content, and DNA content and 132% for myelin content (Table 1). When myelin content was normalized to body weight, brain weight, brain protein content, or brain DNA content, myelin content of transgenic mouse brains at both ages was significantly greater than that of the controls (Table 1), indicating that myelin was increased preferentially and not simply as a consequence of increased brain or body growth, or increased brain cell number. Looking at data from a larger number of litters and time points, the transgenic mice had 15%–36% more myelin per g of brain weight than the controls (significant at $p < 0.02$) at all ages from day 25 to day 82 (Figures 5B and 5C).

It is also instructive to consider the total myelin content of the transgenic mice and ask how much of the total myelin would be expected simply because the brains are larger and how much of it is disproportionate. Simple calculations from the data in Table 1 provide some answers. At day 55, the control mouse brains contain 9.177 mg of myelin. The transgenic mouse brains are 55% larger than the controls (see data for brain weight), and so they would be expected to contain 55% more myelin than the controls, or 14.224 mg total, if myelin content is increased in proportion to brain weight. In fact, the transgenic mouse brains contained 21.313 mg of myelin (Table 1, Brain myelin, mg). Thus, approximately two-thirds of the total myelin of the transgenic mouse brains (14 of the 21 mg observed) would be expected simply because of the size of the brains, and the remaining one-third of the total myelin is in excess of that. The mathematical equivalent is shown in Table 1: myelin/brain weight of the transgenic mice is 49% greater than that of the controls (21.313.../14.224... = 1.49 = 149%). These relationships are also evident when Figure 5B is compared with Figure 5A: even after correction for the difference in brain weight, the transgenic mice have substantially more myelin than the controls.

Similar relationships were observed in the cerebrum, which contains many myelinated structures but is relatively less myelin rich than brain as a whole. Cerebral weight and protein content were significantly increased in the mice, and DNA content showed a small, nonsignificant increase (Table 2). Myelin content of the cerebrum was 123% greater in the transgenic mice than in the littermate controls (Table 2), which was similar to the increase seen in whole brain at the same age (Table 1). When normalized to cerebrum weight, protein content, or DNA content, cerebral myelin content was still substantially greater in the transgenic mice than in the controls (Table 2), which is similar to what was observed in whole brain (Table 1).

CNP Activity Is Elevated in Brains from Transgenic Mice

2', 3'-Cyclic nucleotide 3'-phosphohydrolase (CNP) is present at high activity in oligodendrocytes and constitutes 2%–5% of the total protein of CNS myelin, but is essentially absent from other CNS cells and structures (Kurihara and Tsukada, 1967; Sims and Carnegie, 1978; Vogel and Thompson, 1988; Morell et al., 1989). Because CNP activity can be readily quantified by enzyme activity assay in crude homogenates, thus avoiding errors that might be introduced during the isolation and purification of myelin, CNP activity is a convenient and frequently used index of myelination (Sims and Carnegie, 1978; Vogel and Thompson, 1988; Morell et al., 1989). However, CNP is present in oligodendrocyte cell bodies and processes, as well as in myelin (Vogel and Thompson, 1988; Braun et al., 1988;

Table 2. Weight, DNA Content, and Myelin Content of Cerebrum of IGF-I Transgenic and Littermate Control Mice at Day 56

Measurement	Control, Mean ± SEM	Transgenic, Mean ± SEM	Transgenic, % Greater than Control	p
Cerebrum weight, g	0.222 ± 0.004	0.371 ± 0.017	67	<0.001
Cerebral protein content, mg	20.53 ± 0.97	29.74 ± 0.43	45	<0.001
Cerebral DNA content, arbitrary units	0.808 ± 0.087	0.958 ± 0.100	19	NS
Cerebral myelin content, mg	3.011 ± 0.006	6.727 ± 0.367	123	<0.0005
Myelin/cerebrum weight	13.58 ± 0.26	18.19 ± 1.16	34	<0.02
Myelin/cerebrum protein	0.147 ± 0.006	0.226 ± 0.009	54	<0.005
Myelin/cerebrum DNA	3.82 ± 0.44	7.09 ± 0.39	86	<0.005

Transgenic and control littermate mice were killed at 56 days of age, brains were removed and weighed, and cerebra were dissected, weighed, and homogenized. Aliquots of each homogenate were used for determination of protein, DNA, and myelin content as described in Experimental Procedures. Cerebral myelin content was normalized to cerebrum weight, protein, and DNA content for each mouse individually. Data for control and transgenic mice were compared using Student's t test; NS indicates not significant at $p > 0.30$. The data are from a representative litter with three control and three transgenic mice.

Morell et al., 1989), and so, CNP activity does not reflect myelin content exactly.

CNP activity was assayed in whole homogenates of control and transgenic mouse brains as an independent index of oligodendrocyte development and myelination in the mice. As shown in Figure 6A, total CNP activity was elevated in the brains of transgenic mice as compared with those of the controls, reminiscent of the increase in total myelin content (Figure 5A). Between days 15 and 55, CNP levels increased at a more rapid rate in the brains of transgenic mice than in those of nontransgenic mice. Thus, CNP levels were 87% higher in the brains of transgenic mice than those of the controls by day 55. However, CNP levels increased less rapidly after day 55, so that by day 82, total CNP levels were only 41% higher in transgenic mouse brains than in controls. The specific activity of CNP (U per mg of brain protein) also reached an earlier and higher plateau in transgenic mouse brains than in nontransgenic brains (Figures 6B and 6C). When CNP activity was normalized to brain protein content, activity was still substantially greater in the transgenic mice than in the controls (Figure 6B). When the CNP data were compared within the same litter, the differences in the specific activity between transgenic and control mouse brains were statistically significant at all ages examined ($p < 0.05$)(Figure 6C).

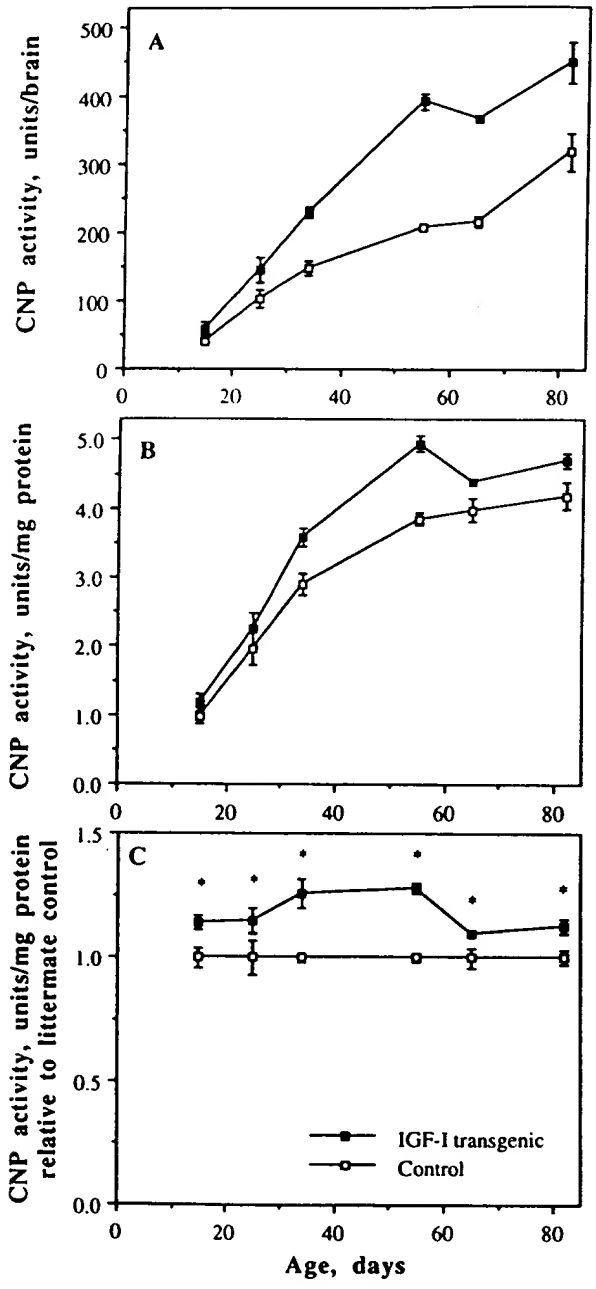


Figure 6. CNP Activity in Brains of Transgenic and Control Mice

(A) Total CNP activity per brain; (B) units of CNP per mg of brain protein; (C) values for each mouse are expressed relative to the mean of the nontransgenic mice from the same litter, as in Figure 5C. Each data point represents a minimum of two experiments and eight mice per condition except for day 65, which represents one experiment and three mice per condition. In (C), * indicates significantly greater than control ($p < 0.05$, Student's t test). Error bars represent SEM; when not shown, the error bars are smaller than the symbols.

Oligodendrocyte Number Is Not Preferentially Increased in IGF-I Transgenic Mouse Brains

Although the differences between the transgenic and the control mice were not statistically significant, DNA assays suggested a 15%-25% increase in total cell number in the transgenic mouse brains (Table 1; data not shown). To determine whether the increased myelin content of the transgenic mouse brains was due to an increase in oligodendrocyte numbers, we counted oligodendrocytes and nonoligodendroglial cells in eight representative brain regions. Cells were counted at day 55 for comparison with measurements of brain size and myelin content at days 55-56. Oligodendrocytes were identified by immunoperoxidase staining for carbonic anhydrase II (CA II), which gives darkly stained oligodendrocyte cell bodies but little staining of myelin, so that oligodendrocytes are not obscured (Ghandour et al., 1980; LeVine and Goldman, 1988). Structures chosen for study included white matter regions with large numbers of oligodendrocytes (anterior commissure, fornix, and corpus callosum), gray matter regions with few oligodendrocytes (superficial gray layer of superior colliculus, central gray, and frontal neocortex), and regions with intermediate numbers of oligodendrocytes (occipital neocortex and dorsal cortex of the inferior colliculus).

The numbers of oligodendrocytes detected by our methods were in general agreement with numbers reported in the literature (Sturrock, 1974, 1976; Gabbott and Stewart, 1987). As a percentage of total cell number, oligodendrocyte numbers were not increased in th transgenic mouse brains in any of the areas studied (Figure 7). Numbers of CA II-positive cells were actually slightly lower in the corpus callosum of transgenic mice as compared with controls (49.2% ± 1.7% versus 55.2% ± 0.9%; $p = 0.04$), whereas oligodendrocyte numbers were not signifi-

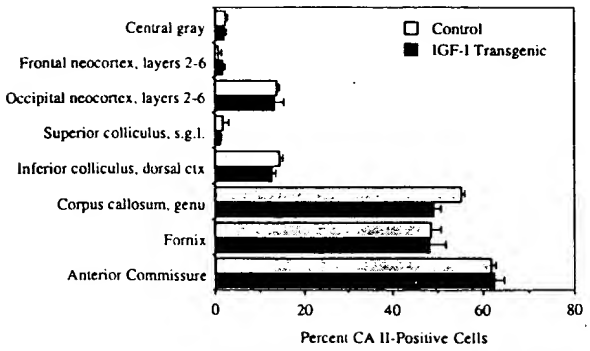


Figure 7. CA II-Positive Cells (Oligodendrocytes) as a Percentage of Total Cell Number in Various Brain Regions of IGF-I Transgenic and Control Mice

Brains were fixed by perfusion at 55 days of age, sections were immunoperoxidase stained for CA II, and cells (omitting vascular cells) were scored in the regions indicated in midsagittal brain sections as described in Experimental Procedures. ctx = cortex; s.g.l. = superficial gray layer. In each brain, 250–1000 cells were scored per brain region. The figure shows the mean ± SEM for five control and five transgenic mouse brains scored in the anterior commissure and the fornix, and three control and three transgenic brains scored in the other areas. Data for transgenic and control mice were compared using Student's t test. The data were significantly different in the corpus callosum ($p = 0.04$); no significant differences were observed in the other seven regions ($p > 0.2$).

cantly different in transgenic versus control mice in the other seven areas studied ($p > 0.2$; see Figure 7). Examination of other areas throughout the brains of transgenic and control mice failed to reveal any substantial differences in the relative abundance of oligodendrocytes (data not shown). Thus, numbers of oligodendrocytes and nonoligodendroglial cells were affected to the same extent in the transgenic mouse brains. Moreover, the nearly 2-fold increase in myelin content per mg of DNA of the transgenic mouse brains cannot be accounted for by an increase in the relative abundance of oligodendrocytes.

In addition, we measured the size of two white matter structures to determine whether oligodendrocyte- and myelin-rich brain regions in the transgenic mice were substantially increased in size at the expense of oligodendrocyte- and myelin-poor regions, which might result in an increase in the total oligodendrocyte number of brains even though relative oligodendrocyte abundance within each region was unchanged. The anterior commissure and the corpus callosum (together with the immediately adjacent dorsal fornix and the dorsal hippocampal commissure) were selected for study because they are both oligodendrocyte rich, they are frequently studied and well-characterized white matter regions, and their shape and orientation make them amenable to measurement of size in midsagittal sections.

The anterior commissure is an approximately cylindrical structure containing a constant number of axons and presenting a uniform size and profile as it crosses the midline. The corpus callosum, together with the immediately adjacent white matter tracts, the dorsal fornix and dorsal hippocampal commissure, forms a well-defined structure that is the largest white matter structure in the cerebrum. Although less regular in shape than the anterior commissure, there is little variation in size of the structure in sagittal sections within 0.1 mm of the midline, which is where we took our measurements. Measurements were made at day 55, at which time the ratio of transgenic/control brain weight (representing size in three dimensions) was 1.55–1.67, so that the cross-sectional area (two-dimensional size) of a given structure in the transgenic brains would be 1.34- to 1.41-fold that of the controls if all structures are enlarged by the same proportion. The cross-sectional area of the anterior commissure of the transgenic mice was 1.37 ± 0.18 times that of the controls (five transgenic and five control mice), within the expected range, whereas the area of the corpus callosum was 1.56 ± 0.07 times that of the controls (three transgenic and three control mice), which is 11%–16% greater than expected.

Discussion

The present study demonstrates that IGF-I substantially enhances brain growth in vivo. Brain growth was more rapid in the IGF-I transgenic mice than in the controls and continued beyond the time that normal brain growth essentially ceases. The brains of the transgenic mice became as much as 70% larger than those of the controls. Many, if not all, CNS structures and cell types appeared to be affected. Grossly and histologically, all brain structures appeared to be enlarged to a similar extent, and gray matter–rich brain regions were increased in weight to nearly the same extent as white matter–rich regions. Measurements of two major white matter structures showed that the anterior commissure was increased in size in proportion to the increase in total brain size, whereas the corpus callosum–dorsal fornix–dorsal hippocampal commissure was increased 11%–16% in excess of proportionality. At the cellular level, the actions of IGF-I appeared to be diverse. DNA assays reported here and elsewhere (Mathews et al., 1988) consistently showed a 20%–25% increase in DNA content in the transgenic mouse brains, although the increase was not statistically significant. However, counts of oligodendrocytes and nonoligodendroglial cells showed that the percentage of oligodendrocytes was not increased in any brain structure, indicating that any increase in total cell number in the transgenic mouse brains affected oligodendrocytes and nonoligodendroglial cells equally. This observation is in agreement with reports that IGF-I is a growth factor for developing CNS neurons and astrocytes (Han t al., 1987; Shemer et al., 1987; Nielsen et al., 1991), as well as for oligodendrocytes (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988; Mozell and McMorris, 1991; Barres et al., 1992).

Other parameters of growth were also affected in the IGF-I transgenic mice. Total body weight was increased by 25%–30%. Mathews et al. (1988) reported that the spleen and pancreas in these mice were approximately doubled in weight owing to a doubling in cell number; and the kidney was increased in weight by approximately 25%. IGF-I levels in the same organs were increased by 2-, 5000-, and 5-fold, respectively. However, not all tissues with elevated IGF-I levels were increased in weight: liver, lung, and heart weights were unaffected, despite increases in tissue IGF-I levels up to 50-fold (Mathews et al., 1988). Whereas brain size was strikingly increased in the IGF-I transgenic mice, the concentration of IGF-I in brain was among the lowest of any tissue measured, and its increase in the transgenic brains—less than 2-fold—was also among the lowest (Mathews et al., 1988). These findings suggest that brain growth is extremely sensitive to IGF-I levels.

Myelination was also increased in the transgenic mouse brains, and the increase was greater than expected from the increase in brain size. The rate of myelination was accelerated throughout the main period of CNS myelination in the IGF-I transgenic mice as compared with their nontransgenic littermates. Consequently, myelin content in the brains of IGF-I transgenic mice was as much as 130% greater than that in brains of the controls, whereas brain weight and brain protein were approximately 50%–70% higher and brain DNA content appeared to be 20%–25% higher in transgenic mice than in the controls. Considering that myelin accounts for approximately 25% of the total dry mass of the mature brain in rodents (Norton, 1981), the 130% increase in myelin content was striking. A similar response was observed for CNP, a marker for myelin and oligodendroglial membranes.

Based on previous studies showing that IGF-I increases the number of oligodendrocytes that develop in vitro, we expected that the brains of our transgenic mice would contain greater numbers of oligodendrocytes. In these prior studies, IGF-I increased the number of oligodendrocytes that developed in glial cell cultures by anywhere from 2-fold (in reaggregate organ cultures) to 60-fold (in monolayer cell cultures maintained in serum-free, growth factor–free culture medium) (McMorris et al., 1986; Mozell and McMorris, 1991). IGF-I increased oligodendrocyte numbers both by acting as a mitogen for oligodendrocytes and oligodendrocyte precursors and by inducing glial precursors to develop into oligodendrocytes (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988). Recently, IGF-I has also been reported to act as a survival factor for oligodendrocytes and oligodendrocyte precursors (Barres et al., 1992). Therefore, we expected to find an increase in oligodendrocyte numbers corresponding to the 2.25-fold increase in myelin content.

Unexpectedly, we were unable to find evidence for a sufficient increase in oligodendrocyte numbers to account for the 2.25-fold increase in myelin content at the same age. Counts of oligodendrocytes in a variety of brain structures, including oligodendrocyte-poor and oligodendrocyte-rich areas, failed to reveal significant increases in the percentage of oligodendrocytes in any of these structures. Additionally, there was no evidence that oligodendrocyte-rich white matter areas were substantially enlarged at the expense of oligodendrocyte-poor areas, which would have resulted in an increase in the overall percentage of oligodendrocytes in the brain, even though their relative abundance within white matter itself was unchanged. The anterior commissure was enlarged in proportion to the enlargement of brain as a whole, and the corpus callosum was enlarged by 11%–16% in excess of proportionality. Some excess enlargement of these structures might be expected because of the space occupied by the additional myelin; this might account for the excess enlargement of the corpus callosum, the adjacent dorsal fornix, and the dorsal hippocampal commissure, but was not detectable in the case of the anterior commissure. (Because the volume of myelin as a percentage of the total volume of white matter or whole brain has never been determined, we cannot predict what actual increase in volume would be expected.)

Thus, as a percentage of total cell number, oligodendrocyte number is similar in the transgenic and control mice. In contrast, per mg of DNA (as a measure of total cell number), myelin content was approximately 85% greater in the transgenic mice than in the controls. In absolute terms, total oligodendrocyte number appeared to be increased by 25%, as indicated by the apparent 25% increase in total DNA content, whereas total myelin content was increased by 125%–130%. Therefore, we conclude that the increase in myelin content is primarily the result of an increase in the amount of myelin produced per oligodendrocyte. Whether this increase is due to an increase in the number of myelin internodes produced per oligodendrocyte or to an increase in the size of the internodes remains to be determined.

The observation that IGF-I greatly increases oligodendrocyte number in vitro but not in our transgenic mice is probably because of a basic difference in the two experimental systems. In previous tissue culture experiments (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988; Mozell and McMorris, 1991), the control cultures lacked IGF-I, insulin, and other exogenous growth factors, and so, the experiments compared conditions of IGF-I depletion and IGF-I restoration. In the current experiments, the controls were normal mice expressing normal tissue levels of IGF-I, and the experimental condition was one of IGF-I overexpression. Conversely, in cases of IGF-I or growth hormon d ficiency in vivo, CNS my lin is greatly reduced, apparently owing to a reduction in oligodendrocyte numbers (Pelton et al., 1977; Noguchi et al., 1982a, 1982b, 1985; Sugisaki et al., 1985; King et al., 1988; M. J. Carson, R. R. Behringer, R. L. Brinster, and F. A. McMorris, unpublished data). Our

finding that there was little increase in oligodendrocyte number when IGF-I was overexpressed in vivo suggests that the increase in CNS IGF-I levels in the transgenic mice was insufficient to increase oligodendrocyte numbers substantially, or that the normal IGF-I levels in the developing mouse brain are sufficient to drive the number of oligodendrocytes essentially to a maximum. Alternatively, compensatory mechanisms that normally coordinate the numbers of different cell types generated in the developing CNS may have prevented a large overshoot from occurring in oligodendrocyte numbers.

Our observation that IGF-I increases the amount of myelin produced per oligodendrocyte indicates that IGF-I regulates myelin production by oligodendrocytes, in addition to regulating the proliferation and differentiation of oligodendrocytes and oligodendrocyte precursors. This finding is in agreement with tissue culture studies which show that IGF-I regulates the amount of CNP produced per oligodendrocyte (R. D. Meyer, N. T. Marchetti, and F. A. McMorris, unpublished data). It is possible that IGF-I acts directly on oligodendrocytes or oligodendrocyte precursors, or alternatively, that the primary target of IGF-I is some other cell type which then interacts with the CNS myelination system in some way, such as by producing another growth factor, to lead to the increase in myelin production. We favor the former interpretation for several reasons. We have previously shown that both oligodendrocytes and oligodendrocyte precursors have cell surface type I IGF receptors—the receptors that mediate the growth effects of IGF-I and IGF-II—as well as type II IGF receptors, whose function is unknown (McMorris et al., 1986, 1990; F. A. McMorris and R. W. Furlanetto, unpublished data). Moreover, IGF-I and IGF-II promote oligodendrocyte development in vitro in a variety of culture systems that vary widely in the numbers and types of nonoligodendroglial cells present, from purified cultures of oligodendrocytes or glial precursors to organ cultures containing large numbers of all major neural cell types (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988; Mozell and McMorris, 1991) and, in the present case, in brain in vivo. These observations are most consistent with direct action on oligodendrocytes and their precursors.

Expression of the IGF-I sequence in the transgene is driven by the mouse metallothionein promoter, which is active in most cell types, including astrocytes (Swanson et al., 1985; Young et al., 1991). Thus, it is likely that many cell types in the transgenic mice produce IGF-I and that its production is not developmentally regulated. IGFs are present in normal brain, but much remains to be determined about the cellular origin and the developmental regulation of the IGF that is available to developing oligodendrocytes. Both IGF-I and IGF-II and their mRNAs have been observed in normal brain in vivo at various developmental ages, including the early postnatal period when oligodendrocyte development and myelination are at their peak (D'Ercole et al., 1984; Brown et al., 1986; Lund et al., 1986; Rotwein et al., 1988; Baskin et al., 1988). Both astrocytes and neurons have been reported to produce IGF-I (Ballotti et al., 1987; Rotwein et al., 1988; Bondy, 1991; Komoly et al., 1992), and the choroid plexus and the meninges are a source of IGF-II (Stylianopoulou et al., 1988; Hynes et al., 1988), which then circulates in the cerebrospinal fluid. IGFs also enter the brain from the peripheral circulation (Hodgkinson et al., 1991). Presumably, IGF-I or IGF-II from some of these sources is available to oligodendrocytes, while IGFs from other sources might perform other functions in the CNS. IGF-binding proteins, which can dramatically alter the biological activity of IGF-I and IGF-II, are also present in the CNS and are likely to play an important regulatory role (Lamson et al., 1989; Wood et al., 1990; Ooi, 1990). Finally, the timing of IGF-I expression during development is likely to be very important. Various treatments greatly affect CNS myelination when administered during the first few postnatal weeks in rats and mice, but are ineffective at later times, suggesting the concept of a "critical period" for CNS myelination (Wiggins, 1982; Sugisaki et al., 1985). Each of these parameters represents a point at which the effects of IGFs on myelination might be modulated.

In summary, we have shown that IGF-I is a potent inducer of brain growth and especially of myelination in the CNS in vivo. These findings clarify the role of IGF-I in vivo, provide insight into the regulation of CNS development and myelination, and may prove useful in designing treatments for myelin disorders and demyelinating conditions.

Experimental Procedures

Mice

The transgenic mouse line used in these studies, designated Tg(Mt-1,IGF-I)Bri45 (previously known as line 1219-6), has been described (Mathews et al., 1988). Mice were propagated by mating hemizygous transgenic males with control females, resulting in litters containing transgenic and nontransgenic siblings in approximately a 1:1 ratio. Transgenic progeny were identified by dot blot hybridization of tail DNA as described (Brinster et al., 1985). Nontransgenic littermates were used as controls in all experiments to compensate for differences in litter size, nutrition, general health, maternal behavior, etc., which can affect the rate and extent of myelination (Wiggins, 1982). Whenever possible, transgenic and nontransgenic mice were sex matched within a single experiment. However, the accumulated data showed that gender had no effect on the parameters measured in these experiments.

Myelin Isolation

Myelin was isolated by sucrose density gradient centrifugation as described by Norton and Poduslo (1973). Briefly, mice were sacrificed by carbon dioxide inhalation, and the brains were removed, weighed, partially cleaned of meninges, homogenized in 0.32 M sucrose, and layered over an equal volume of 0.85 M sucrose. After centrifugation for 30 min at 82,500 × g, myelin was collected from the interface, pelleted, and water shocked for 30 min to remove matter trapped within myelin vesicles. The myelin was centrifuged again on a 0.32 M, 0.85 M discontinuous sucrose gradient, collected from the interface, and washed four times with water to remove sucrose. The final myelin fraction was lyophilized in tared tubes and weighed.

Assay of CNP Activity

An aliquot of the brain homogenate was frozen on dry ice immediately after homogenization and stored at −70°C until the time of the assay. After thawing, the homogenates were sonicated, and CNP activity was assayed by the method of Prohaska et al. (1973), using toluene:isobutanol (1:1) instead of benzene:isobutanol (1:1) to extract the colored phosphomolybdic acid complex. A unit of CNP activity is defined as the number of micromoles of adenosine 2', 3'-cyclic monophosphate converted to adenosine 2'-monophosphate per min. CNP-specific activity is expressed as U of CNP per milligram of protein.

Protein and DNA Determinations

Protein and DNA were assayed in whole homogenates of brain tissue. Protein determinations were made using the bicinchoninic acid modification (Smith et al., 1985) of the method of Lowry et al. (1951), with bovine serum albumin as the standard. DNA content was assayed by the Hoechst 33258 dye binding assay of West et al. (1985) and read in a spectrofluorimeter at an excitation wavelength of 350 nm and an emission wavelength of 455 nm. Salmon sperm DNA was used as the standard.

Determinations of the Water Content of Brain

Brains were removed from mice sacrificed by carbon dioxide inhalation, rapidly weighed in tared containers, and flash frozen in liquid nitrogen. Brains were subsequently lyophilized for 4 days and reweighed.

Histology

Mice were anesthetized with Metofane (Pitman-Moore) and perfused with ice-cold phosphate-buffered saline, followed by 4.5% paraformaldehyde in 0.1 M sodium phosphate (pH 7.5) through the heart. Brains were then removed from the cranium, sliced sagittally with a razor blade, and placed in the same fixative at 4°C for an additional 48 hr. Tissue was then embedded in paraffin and sectioned at 6 μm. For immunostaining, sections were deparaffinized, rehydrated, and stained by the avidin-biotin complex immunoperoxidase method (Hsu et al., 1981), using rabbit antiserum to CA II (1:300) (a gift from Drs. S. Ghandour and R. Skoff) and reagents purchased from Vector Laboratories, Burlingame, CA (Vectastain Elite). Sections were then counterstained with hematoxylin and mounted.

Cell Counting

Sections were viewed under a Leitz Orthoplan microscope, and images were projected onto a color monitor via a color video camera. A positively stained cell was defined as a hematoxylin-stained nucleus surrounded by darkly immunoperoxidase-stained cytoplasm. In initial experiments, scoring was done by tracing the perimeter of the structure in each section from the video monitor and then marking and scoring each cell on the tracing. Subsequently, scoring was done using a camera lucida device (Microvid, Leitz) that allowed viewing of the cells under the microscope superimposed on the image of a computer-generated grid. Each cell was scored visually and marked on the computer display using software developed in this laboratory (Cell Count 2.0), which then tallied the numbers and positions of the scored cells. To avoid double counting of the same cell in consecutive tissue sections, only sections that were greater than 50 μm apart were scored. The cross-sectional area of the anterior commissure and of the corpus callosum with the immediately adjacent dorsal fornix and dorsal hippocampal commissure was determined in sagittal sections within 0.1 mm of the midline. The outline of the structure was traced on a plastic sheet on the video monitor. The tracing was then photocopied, and the area was cut out and weighed. Scoring of the anterior commissure and the fornix was done on five transgenic and five littermate control mice; scoring for other structures was done on three transgenic mice and three littermate controls. For each mouse, 250–1,000 cells were counted per structure and area determinations were done on 2–3 sections per structure.

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491.11

EXPRESSION OF NCAM PSA IN THE HUMAN HIPPOCAMPAL DENTATE GYRUS FROM INFANCY TO OLD AGE. G.B. Fox*, C.M. Ni Dhuill, S.J. Pittock, A.W. O'Connell and C.M. Regan, Department of Pharmacology, University College, Belfield, Dublin 4, Ireland.

The polysialylated form of the neural cell adhesion molecule (NCAM-PSA) regulates adult neuroplastic events associated with learning and memory. Although perturbations of NCAM function have been related to the severity of the impaired sensory processing associated with autism and schizophrenia, few studies have examined the distribution of NCAM-PSA in neuroplastic regions of the human brain. We now describe the distribution of polysialylated NCAM in the aging human hippocampal dentate gyrus, a region synonymous with processing learning-associated sensory information. Human brain tissue was obtained, with the consent of the senior pathologist, from six major Dublin hospitals within 24 hours post-mortem. The hippocampus was isolated from 16 individuals ranging in age from 5 months to 82 years with at least one sample from each decade. The tissue was dissected from the medial temporal lobe, coated in optimal cutting temperature compound and frozen in liquid nitrogen- or dry ice-cooled n-hexane. Cryosections of the dentate area were prepared and immunostained for NCAM-PSA using a monoclonal antibody specific for the polysialic acid portion of the molecule. Immunoreactivity was localised mainly to neurons in the granule cell layer and their mossy fibre axons of the infant (5-7 months). In early childhood (1-3 years), this immunoreactivity began to decline and was markedly reduced in old age. In contrast, a population of large neurons in the hilar region became immunoreactive only during early childhood and remained in significant numbers into adulthood and old age. These results demonstrate a differential, age-dependent expression of NCAM-PSA in two discrete neuronal populations of the human hippocampal dentate gyrus. *Supported by the Health Research Board of Ireland and the EU Biotechnology Programme.*

491.12

DIFFERENTIAL EXPRESSION OF Na-K ATPase α-ISOFORM mRNAs IN AGING RAT CEREBELLUM. N.B. Chauhan*, G.J. Siegel, Molecular and Cellular Neuroscience Laboratory, Hines VA, Hines IL 60141; Departments of Neurology, Molecular and Cellular Biochemistry, Loyola University Chicago, Maywood IL 60153.

Age-dependent changes in the expression of Na,K-ATPase α1- and α3-mRNAs were analyzed in the rat cerebellum by *in situ* hybridization. In young rats, α1-mRNA showed prominent labeling in the granular layer (GL) with moderate fine distribution in the molecular layer (ML), Purkinje cell layer (PCL) and white matter (WM) but no clusters over Purkinje cells. In old rats, α1-mRNA remained unchanged in ML and PCL but declined by 43% ($p<0.0001$) in GL and increased by 624% ($p<0.0001$) in WM. α3-mRNA in young rats showed large clusters of label on stellate, basket, Golgi and Purkinje cells (PCs) and fine grains diffusely in ML, GL and WM. In old rats, α3-mRNA declined by 87% in ML, 83% in PCL, 84% per PC, and 89% in GL and increased by 111% in WM (all values $p<0.0001$) relative to young rats. PC numbers were reduced by 30% but the average area of PC profiles did not change significantly. In old rats, the specific cluster-like label related to α3-mRNA on PCs, stellate, basket and Golgi cells was lost. Immunocytochemistry of cerebellum and hippocampus showed no age-related change in the distribution and density of total catalytic polypeptide. Thus, the discordance between changes in the levels of mRNAs in neuronal layers and WM in the face of constant polypeptide levels indicates age-related changes also in polypeptide turnover. Cell- and isoform-specificity of α-isoform mRNAs in aging rat cerebellum may reflect differential regulation underlying age-related impairments in signal transduction and motor learning.

491.13

DECREASES IN TYPE 1 INSULIN-LIKE GROWTH FACTOR RECEPTORS IN CORTEX AND HIPPOCAMPUS OF AGED RATS. P. Thornton*, S. Bennett, P. Cooney, R. Ingram and W. E. Sonntag, Department of Physiology and Pharmacology, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27157-1083.

IGF-1 has previously been shown to have an important role in brain function including protein synthesis, neurite outgrowth, release of acetylcholine and cortical development in rodents. In this study, we assessed whether a decline in the type 1 IGF receptor could be a contributing factor in the decrease in brain function that occurs with age. Brain sections from Fisher 344xBrown Norway male rats (10, 19 and 29 months of age) were assessed for ^{125}I-IGF-1 and ^{125}I-des[1-3]IGF-1 recept r binding by autoradiography. Type 1 IGF receptors were prominent in both layers 2 and 4 of sensorimotor cortex and in hippocampus of all animals. ^{125}I-IGF-1 binding tended to increase in layers 2 and 4 from 10 to 19 months of age and decreased significantly from 19 to 29 months in layer 2 (3.65±0.2 vs 2.76±0.2nCi/mg, $p<0.05$) and layer 4 of cortex.(3.20±.0.15 vs 2.42±0.22nCi/mg). In hippocampus, receptor density decreased steadily from 10 to 29 months (3.66±0.22 vs 2.48±0.15nCi/mg, $p<0.05$). Analysis of adjacent sections with ^{125}I-des[1-3]IGF-1 revealed similar decreases with age in layer 2 (35% decrease from 19 to 29 months) and 4 (33% decrease from 19 to 29 months) of cortex. Binding in hippocampus decreased by 25% from 10 to 29 months of age. The results of these studies demonstrate an age-related decline in type 1 IGF receptor density and suggest that these decreases may have a role in the decline in brain function with age.

Supported by NIH grant 1 P01 AG11370.

AGING PROCESSES

492.1

AGE-DEPENDENT CHANGES OF THE LEVELS OF FACTORS INVOLVED IN CHOLESTEROGENESIS OF RAT CENTRAL AND PERIPHERAL NERVOUS SYSTEMS. R. Fukuyama[a], S. Fushiki* and K. Yanagisawa[a]. [a]Dept. Dementia Res., National Inst. Longevity Sci., Obu, Aichi, 474; Dept. Dynamic Pathol., Res. Inst. Neurol. Dis. Geriatrics, Kyoto Prefect. Univ. of Med., Kamigyo, Kyoto, 602 Japan.

A major risk factor for Alzheimer's disease (AD) is gene dose of ε4 allele of apolipoprotein E (ApoE), which is assumed to function as a carrier of phospholipids, cholesterol and triacylglycerol. It may be a clue to determine whether *de novo* biosynthesis or recycling system is dominant for supplying cholesterol in adult brain, in order to understand molecular pathogenesis of AD. We determined the levels of mRNA and protein of hydroxy methylglutaryl CoA-reductase (HMGR), the rate-limiting enzyme in cholesterogenesis, for evaluation of endogenous supply of cholesterol and also determined those of ApoE for evaluation of recycling cholesterol at various ages of rat brain hemisphere and sciatic nerve. Quantity of mRNA of HMGR was estimated by simplified RT-competitive PCR technique. We also measured total cholesterol with enzymatic procedure in order to relate the levels of these molecules to concentration

492.2

AGE RELATED CHANGES IN GLUTAMATE RECEPTORS IN THE CANINE BRAIN CORRELATE WITH CHANGES IN ANESTHETIC POTENCY C.D. Rinehart[1*], C. Dunlop[2], A. Wagner[2] and K.R. Magnusson[1]. Depts. f Anatomy & Neurobi l gy[1] & Clinical Sciences[2]; Colorado State University, Fort Collins, CO 80523.

With increasing age, animals and humans become more sensitive to the depressant effects of general anesthesia. Antagonists of NMDA and AMPA receptors can also increase the potency of anesthetics. The goal of this study was to determine whether dogs experience a reduction in the functioning of glutamate receptors with age that could thereby impact the anesthetic requirement of the animal. Six young (2-3 years) and 6 middle aged (11 years) beagles were anesthetized with isoflurane to determine the potency (MAC value) for each animal. Following euthanasia, quantitative autoradiography for NMDA and AMPA receptors was performed. The sections were incubated with either 20nM ^{3}H-AMPA or 100nM ^{3}H-glutamate in the presence of

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AGING-RELATED CHANGES IN IGF-II AND C-FOS GENE EXPRESSION IN THE RAT BRAIN

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Abstract—The protein products of growth factor genes such as IGF-II and cellular oncogenes such as c-fos are believed to be necessary for the support of normal neuronal function. Steady-state levels of c-fos and IGF-II mRNA were determined in the brain of young and old rats, using Northern analysis. Both RNAs were found to be decreased in the brain of aged rats. Age-related decrease was detected in the hippocampus, hypothalamus, striatum, cerebral cortex and cerebellum, for IGF-II mRNA, and in the cerebral cortex and cerebellum for c-fos mRNA. Furthermore, changes in the degree and pattern of DNA methylation were noted at both gene loci, in the aged rat brain. Our results could reflect changes at the genomic level possibly related to the process of aging and the accompanying decline in brain function.

Key words: aging, aged brain, IGF-II, c-fos, mRNA, DNA methylation.

Aging is accompanied by a number of biochemical,[14,27] electrophysiological[8] and morphological[4] changes in the rodent brain. Learning, memory[8] and *in vitro* paradigms of neuronal plasticity such as kindling[12] are known to decrease as a result of senescence. These changes are believed to be the end result of altered gene expression.

The protein products of growth factor genes and cellular oncogenes are believed to be involved in the molecular mechanisms underlying neuronal plasticity.[39] We thus decided to investigate IGF-II (insulin-like growth factor II) and c-fos gene expression in the aged as compared to the normal adult rat brain.

IGF-II gene expression stops or is very low in all tissues of the adult rat with the exception of the brain[25,41] where transcription is localized in the choroid plexus and the leptomeninges.[1,42] IGF-II RNA and protein levels are quite high in the choroid plexus[42] and the CSF,[18] respectively. Furthermore, the IGF-II protein,[19] as well as its receptor,[44] have been found in the brain. Moreover, IGF-II has been shown to stimulate the growth rate of normal astroblasts in culture,[24] and to enhance neurite outgrowth in human neuroblastoma cells[35] and in primary cultures of sensory and sympathetic neurons from embryonic chick ganglia.[36] In addition to promoting neurite outgrowth, IGF-II increases tubulin mRNA levels in neuroblastoma cells in culture.[31] Thus IGF-II has neurotrophic properties similar to those of NGF. Relevantly, NGF mRNA, NGF itself,[23] and its receptor[22] levels have been shown to decrease in the aged rat brain.

The protooncogene c-fos encodes for a nuclear phosphoprotein,[6] which after forming a complex with the protein product of another oncogene c-jun, binds to the AP-1 sites of DNA, and acts as a regulatory factor for gene transcription.[5] c-fos mRNA[32] and protein[10] have been found in mammalian neurons, but the levels are low in control situations. However, c-fos is quickly and transiently induced in nervous tissue, as a result of a variety of stimuli.[11,16,17,20,26,33] It has been proposed that c-fos gene expression can be considered as a marker for neuronal activity.[37] Moreover, its protein is believed to be involved in processes related to neuronal plasticity.[39]

Based on the above, we determined using Northern analysis, the steady-state levels of IGF-II and c-fos RNA in the aged compared to control adult rat brain. In addition, we investigated the degree of DNA methylation at the IGF-II and c-fos gene loci, since methylation is believed to be a factor regulating gene expression.[9]

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EXPERIMENTAL PROCEDURES

Animals

Young (3–5 months) and old (18–20 months) male Wistar rats were maintained under controlled temperature (25°C) and lighting (12 hr light–dark cycle). Food and water were available *ad libitum*. The animals were killed by decapitation under light ether anaesthesia, and brains were immediately removed and dissected on ice.

Northern blot analysis

Total RNA was isolated by a single step extraction using the acid guanidinium thiocyanate-phenol-chloroform extraction method.[3] The amount of RNA was determined by absorbance at 260 nm. Northern analysis was performed as previously described.[7] Forty microgram samples of denatured total RNA from various brain areas were electrophoresed on formaldehyde/1% agarose gel, containing 0.4 g/ml ethidium bromide. After electrophoresis and destaining the integrity and quantity of the RNA samples on the gel was verified by visualization and photography under UV illumination. RNAs were then transferred overnight onto Genescreen Plus membrane (New England Nuclear), using 10 × SSC (1.5 M sodium chloride, 150 mM sodium citrate, pH 7.0) as a transfer solution. The membranes were then baked *in vacuo* for 2 hr at 80°C to reverse the formaldehyde reaction, and underwent the same hybridization procedure and post-hybridization washings as previously described.[7] Membranes were exposed to Kodak X-AR 5 film at −80°C with intensifying screen and films were developed in an Agfa automatic developer. Sizes of RNA species were determined in reference to the positions of the 28S and 18S rRNAs. The accuracy of these measurements was verified in control experiments by parallel electrophoresis of a labelled DNA marker (λ phage DNA digested with Hind III).[41] The intensity of each band was quantified according to the following procedure: the positions on the film where the bands were located were cut out in small (10mm × 2–5 mm) strips. Each of these strips was soaked in 1.5 ml 5 N NaOH, 30% in glycerol, until all the silver grains had been eluted off the film. Absorbance was then immediately measured at 500 nm. The ratio between the absorbance value of the control, relative to the aged sample of each Northern analysis was calculated, and means plus or minus standard deviations were determined. In addition, comparisons between the control and aged groups were done using Student's paired *t*-test on ln transformed values of the absorbances.

Southern blot analysis

Genomic DNA was extracted by the phenol method[28] from whole brains of young and aged animals. Twenty microgram samples of genomic DNA from each animal group were mixed with 500 ng of λ DNA and digested with either the restriction enzyme Hpa II or Msp I (Boehringer). The λ DNA was added as an internal marker in order to (a) confirm the completion of the digestion and (b) determine the sizes of the DNA fragments. The digested DNAs were size fractionated by electrophoresis through a 1.8% agarose gel. Following electrophoresis, the DNAs were denatured by placing the gel in an alkaline solution (TS: 0.4 M NaOH, 0.6 M NaCl) for 30 min, with gentle agitation, at room temperature. They were then transferred overnight onto a Genescreen Plus membrane, using the alkaline TS as the transfer solution. After transfer, the membrane was placed in neutralizing solution (0.5 M Tris–HCl, pH 7, 1 M NaCl) for 15 min, and dried for about 1 hr at room temperature. Hybridization, post-hybridization washing conditions, exposure to film and developing were the same as those used in Northern analysis. After determining the restriction pattern at the IGF-II and c-fos loci, each membrane was rehybridized with labelled λ DNA as a probe, after washing off the previously used probe for 30 min at 100°C with 0.1 × SSC, 1% SDS. Every Northern and Southern analysis was carried out in triplicate.

Synthesis of molecular probes

DNA probes were prepared by randomly primed synthesis[13] using the appropriate DNA template, α-^{32}P-dCTP (3000 Ci/mmol, New England Nuclear) and a random primed DNA labelling kit, containing hexanucleotides as primers, deoxyribonucleotides, and the Klenow enzyme (Boehringer). The DNA used as the template to prepare the IGF-II probe was a 545 bp coding

region fragment from rat IGF-II cDNA clone 27,[41] subcloned into the BamH1 and EcoR1 sites of the pGEM-1 vector polylinker. The equivalent DNA for c-fos was a 2188 bp fragment extending from +432 to +2620, and containing the second, third and fourth exons, as well as the intervening introns of the human c-fos gene. It was isolated from a genomic clone of human c-fos in pBR322 by Nae I digestion. The equivalent DNA for β-actin was a 600 bp coding region fragment isolated from pBR322 by Pst I digestion.

RESULTS

Northern analysis

The IGF-II coding region probe used hybridized to the same known[41] multiple transcripts sized 4.5, 2.9, 1.7 and 1.1 kb in brain RNA from both control and aged rats (Fig. 1). The hybridization signal was, however, less intense in the RNA samples prepared from the brain areas of aged rats (Fig. 2.I). Decreased level of IGF-II RNA was observed in all brain areas examined: in the hippocampus, the hybridization signal in the control samples was 2.41 ± 0.68 higher than in the aged; in the hypothalamus the equivalent value was 2.19 ± 0.09; in the striatum 1.94 ± 0.36, in the cerebral cortex 1.92 ± 0.31, and in the cerebellum 1.95 ± 0.30. All the above differences were statistically significant at least at the level of $P < 0.05$. The level of β-actin mRNA was the same in the two age groups, for all the brain areas examined (Fig. 2.II). Furthermore, the amount of RNA loaded was the same in all slots, as verified by ethidium bromide staining of the gel (Fig. 2.III).

When the same membranes were hybridized with the c-fos probe, the level of c-fos RNA was also found to be decreased in the RNA samples prepared from the brain of aged rats (Fig. 3): the characteristic 2.2 kb c-fos transcript was 1.96 ± 0.11 more intense in the cerebral cortex from the control than in the equivalent sample from the aged rats. In the cerebellum, the intensity of the signal was 2.38 ± 0.56 times higher in the control samples than in the aged. These differences were statistically significant ($P < 0.05$, at least). All the brain areas mentioned above in relation to IGF-II were also examined for c-fos RNA. However, the hybridization signal was generally so low that only in the cerebral cortex and the cerebellum were we able to obtain reproducible and reliable results.

Fig. 1. Northern analysis of total RNA (40 μg) isolated from whole brain f control (C) and aged (A) rats. Arrows indicate the positi ns of 28S and 18S rRNAs.

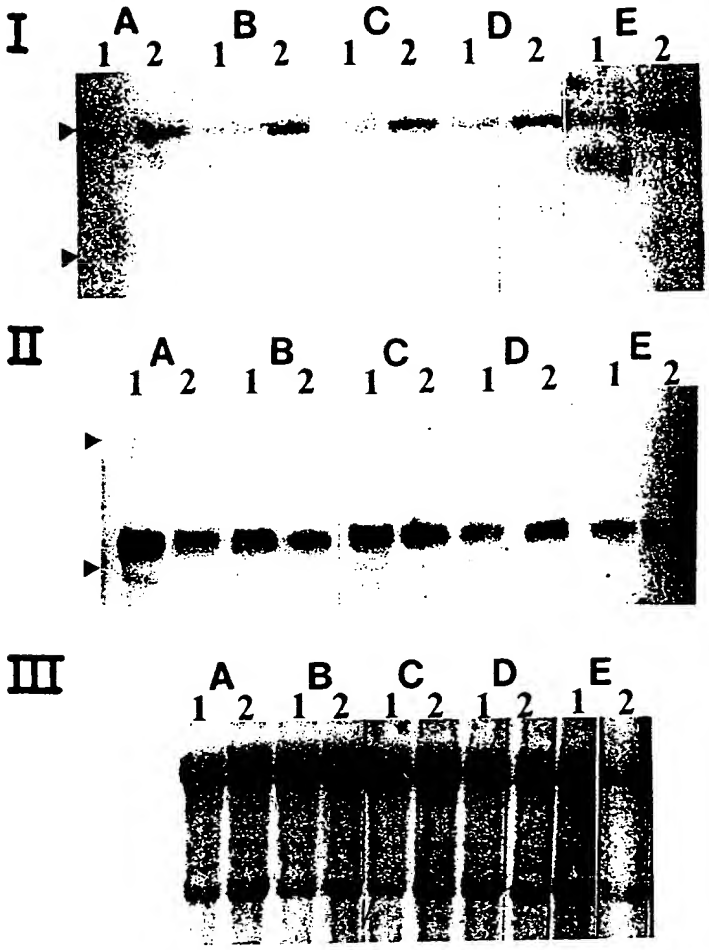

Fig. 2. Northern analysis of total RNA (40 μg) isolated from the hippocampus (A), the hypothalamus (B), the striatum (C), the cerebral cortex (D) and the cerebellum (E) of aged (1) and control (2) rats. I. Hybridization with the IGF-II probe. II. Hybridization with the β-actin probe. III. Ethidium bromide staining of the gel. Arrows indicate the positions of 28S and 18S rRNAs.

Southern analysis

We determined the restriction fragments obtained after Hpa II or Msp I digestion of genomic DNA isolated from aged and adult rat brain. Hpa II and Msp I are isoschizomers which both act on the CCGG sequence, but Hpa II is resistant to mCG, while Msp I is not.[21] We could thus infer about aging related differences in brain DNA methylation, since in mammalian cells the major methylation site is at C of a CG.[9]

The larger-sized bands of the Msp I-digested DNA from control rats consistently migrated faster in the electrophoresis (Figs 4 and 5, first lane). This finding was repeatedly obtained in the different Southerns. It can be attributed to minimization of hindrance of migration due to size, in this sample, since it contains fewer large-sized DNA fragments. In any case, since λ DNA was included as an internal marker in all samples, the sizes of the bands could be accurately determined. The internal marker was also used to verify the completion of the digestion, by examining the Southern blot pattern of λ DNA (data not shown).

Genomic DNA isolated from the brain of control rats, and hybridized with the IGF-II coding region probe, gave seven bands of 3800, 3200, 2750, 2550, 1950, 1630 and 350 bp, when digested with Hpa II (Fig. 4). In the Msp I digest, the 3800 bp and 2750 bp bands were missing, while the 3200 bp and 2550 bp were wider, indicating that they represented a larger number of fragments (Fig. 4). In contrast, the band pattern was identical in the Msp I and Hpa II digests of the genomic DNA isolated fr m the brain f aged rats: in both there were seven bands sized: 4100 (broad), 3200, 2750, 2550, 1950, 1630 and 350 bp (Fig. 4). These results show that the pattern and degree of methylation f the DNA at the IGF-II gene locus is different in the aged as compared to the control adult rat brain.

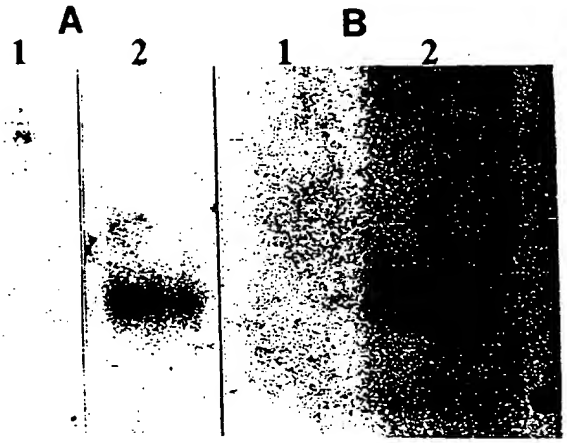

Fig. 3. Northern analysis of total RNA (40 μg) isolated from the cerebral cortex (A) and the cerebellum (B) of aged (1) and control (2) rats, and hybridized with the c-fos probe. Dots indicate the positions of 28S and 18S rRNAs.

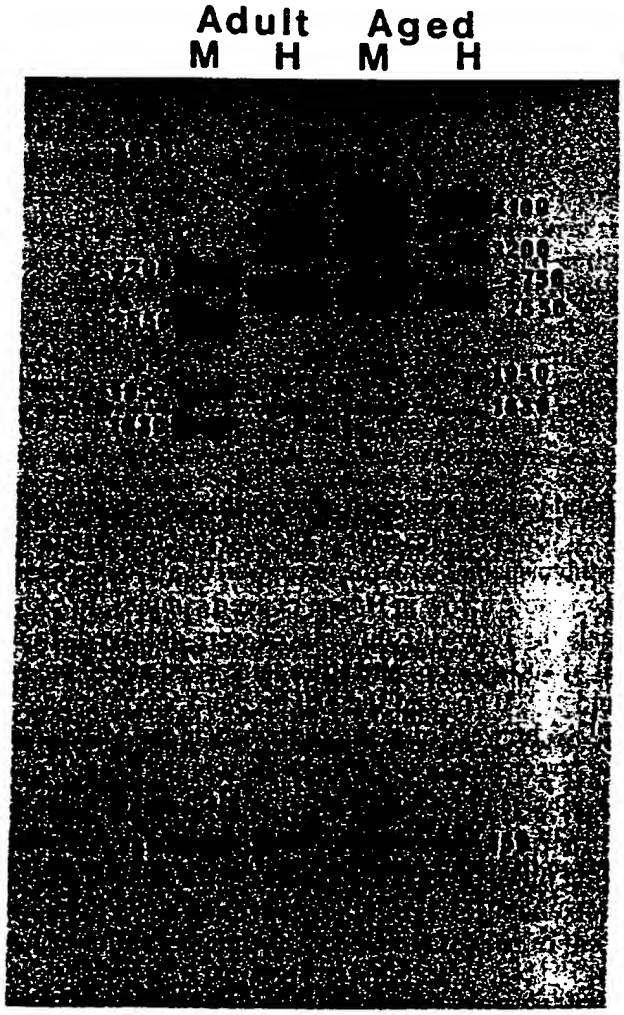

Fig. 4. Southern analysis of genomic DNA (20 μg) isolated from whole brain of control adult or aged rats, digested with either Msp I (M) or Hpa II (H) and hybridized with the IGF-II probe. Numbers represent the sizes of the DNA fragments in bp.

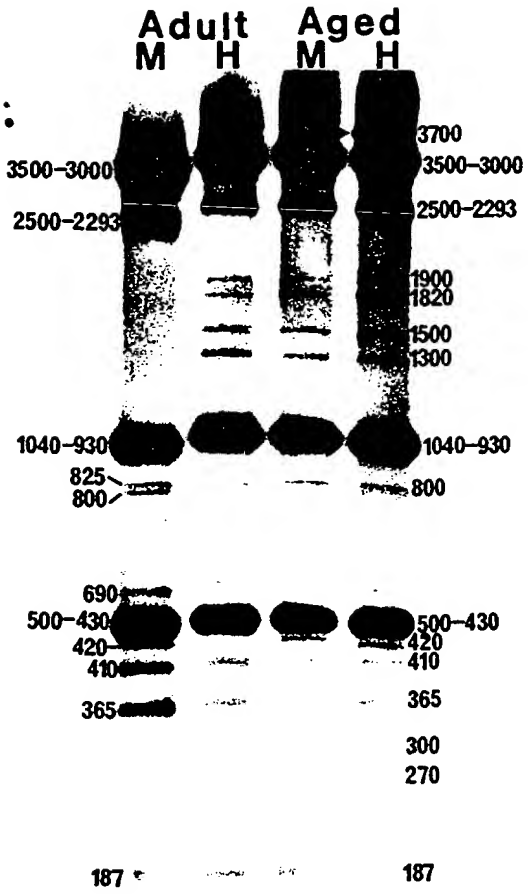

Fig. 5. Southern analysis of genomic DNA (20 μg) isolated from whole brain of control adult or aged rats, digested with either Msp I (M) or Hpa II (H) and hybridized with the c-fos probe. Numbers represent the sizes of the DNA fragments in bp.

Comparison of the band pattern obtained after Southern analysis of Msp I or Hpa II digested genomic DNA hybridized with the c-fos probe, showed that in the samples from aged rat brain the two patterns were indistinguishable, with the exception of a 3700 bp band present only in the Hpa II-digested sample (Fig. 5, note arrow in H lane of aged sample). In contrast, in the samples from the control adult animals the following differences were observed. In the Msp I-digested DNA there was (a) a doublet at 800–825 bp, and (b) a faint band at 420 bp, while in the Hpa II digest there was only one band at 800 bp, and the 420 bp band was missing. Furthermore, the smaller bands sized 410 bp and 365 bp were of greater intensity in the Msp I-digested sample, suggesting that they represented a larger number of fragments. On the other hand, in the Hpa II-digested DNA there were two doublets of bands, one sized 1900 bp and 1820 bp, and the other 1500 bp and 1300 bp, which were absent in the Msp I digest. Taken together these results show that aging is accompanied by changes in the degree and pattern of DNA methylation at the c-fos gene locus in the rat brain.

DISCUSSION

IGF-II gene expression continues at high levels in the brain of the adult rat, while it stops in most other adult tissues.[25,41] This fact, along with the *in vitro* neurotrophic properties of IGF-II,[24,31,35,36] indicates that it is a growth factor important for brain function. However, the exact functional role of IGF-II in the adult rat brain remains to be elucidated. Being synthesized in the choroid plexus[1,42] it could gain access to the brain through the CSF, where it circulates at

high levels.[18] In the brain it could act in a neurotrophic manner analogous to that of NGF. It is quite interesting that like NGF,[23] IGF-II mRNA levels were found to decrease in the aged rat brain. The decrease in IGF-II mRNA was widespread, occurring in brain areas with diverse functions: it was found in areas controlling cognitive functions such as the cerebral cortex and the hippocampus, as well as in the striatum, part of the extrapyramidal motor pathway, the cerebellum, involved in motor functions, and the hypothalamus, the regulatory center for neuroendocrine integration. It has been suggested that aging as well as neurodegenerative diseases, such as Alzheimer's, may be related to a deficient supply or a reduced utilization of trophic agents.[45] Furthermore, NGF has been shown to have ameliorative effects on aging-impaired biochemical[46] and behavioral[15] parameters. In addition to the well documented role of NGF in these processes, IGF-II could also be a participating factor.

In addition to the IGF-II mRNA, steady-state levels of the protooncogene c-fos mRNA were also found to be decreased in the cerebral cortex and the cerebellum of the aged rat brain. This finding could reflect senescence-induced decline in brain function, since c-fos is considered a molecular marker for neuronal activity.[37] It is worth noting that in the rat superior cervical ganglion aging is accompanied by an increase in c-fos immunoreactivity of the sympathetic neurons.[47] However, in the rat SCG aging leads to increased functional sympathetic neuron activity,[2,47] in contrast to the brain, where functions are impaired.[4,8,12,14,27] Thus our findings are compatible with the previous published result on the SCG,[47] and both can be explained on the basis of c-fos function in neurons.[37,39] Moreover, in an *in vitro* system, it has been shown that cellular senescence is accompanied by a repression of c-fos transcription in human fibroblasts.[38]

We have shown decreased steady-state levels of IGF-II and c-fos mRNA in the aged rat brain. These findings can be due to either decreased synthesis, or increased breakdown of the mRNAs. Our data do not permit clarification of this point.

Our results from the Southern analysis showed that at both the IGF-II and the c-fos gene loci of the DNA from the control rats, the Msp I digest contained more smaller-sized bands than the Hpa II digest. This was expected, since Msp I is not resistant to methylation of the internal cytosine of the 5'CCGG sequence (CmCGG), while Hpa II is.[21] The finding that in the DNA from the aged rats the band pattern was basically the same in both Msp I and Hpa II digests can be explained by assuming that aging is accompanied by an increase in the degree of DNA methylation at the outer cytosine of the same target sequence (5'mCCGG), making it resistant to both Msp I and Hpa II digestion.[21] The presence of methylation at the outer C of the CCGG sequence has already been reported in the liver of old rats.[34] It is possible that an increase in methylation at the IGF-II and c-fos gene loci of the DNA in the aged rat brain is related to the decreased levels of the respective mRNAs, observed in this study.

Aging-related changes in the degree of DNA methylation have been reported for a variety of genes in different tissues: c-myc is hypomethylated in the spleen, and hypermethylated in the liver of the aging mouse;[34] vitellogenin in the rooster[29] and intracisternal A particle in mice[30] show hypomethylation in the aging liver; the albumin gene shows an increase in methylation in the liver of the aged rat.[40] As far as the brain is concerned, no change was detected in either the mouse c-myc,[34] or the c-fos[43] gene loci after aging. Our results are not inconsistent with those of the others,[43] since two different regions of the gene were examined in the two papers: we looked at a 2188 bp region of the gene, extending from +432 to +2620 and comprising the second, third, and fourth exons, along with the intervening introns. Uehara *et al.*[43] looked at a region located further downstream: its 5' end was located approximately at the 3' end of the region we examined. It is possible that the senescence-related changes in the degree of methylation at the c-fos locus are restricted only to the region of the gene we examined, and that they do not extend further downstream. The observed changes in DNA methylation at the IGF-II and c-fos gene loci in the aged rat brain could reflect changes at the genomic level possibly related to the process of aging.

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492.15

INSULIN-LIKE GROWTH FACTOR-1(IGF-1) INCREASES WORKING MEMORY IN AGED ANIMALS. M.Mooney, W.E.Sonntag*, M.Barra, X.Xu, S.Bennett, R.Ingram, B.Poe and A.L.Markowska. Department of Psychology, Johns Hopkins University, Baltimore, MD 21218 and Department of Physiology and Pharmacology and Program in Neuroscience, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27157.

The age-related reduction in tissue protein synthesis appears to result, at least in part, from a decline in growth hormone secretion and a subsequent decline in IGF-1. IGF-1 has been implicated in differentiation of cortical neurons and regulation of acetylcholine release in hippocampal neurons. The present study was designed to assess whether behavioral deficits with age may be partially overcome by increasing levels of IGF-1. Fisher 344xBrown Norway male rats (4 months old and 32 months old) were preoperatively trained in behavioral tasks and subsequently implanted with osmotic minipumps to infuse IGF-1 (50ng/hour) or vehicle, icv. Animals were retested at 2 weeks and 4 weeks after surgery. IGF-1 improved performance in the repeated acquisition task. Mild improvements in some measures of the place discrimination task which assesses reference memory were also observed. IGF-1 had no effect on sensorimotor skills but reversed some age-related deficits in emotionality. These data indicate a potentially important role for IGF-1 in the reversal of cognitive impairments that occur in aged rodents.

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492.16

THE EFFECTS OF INCREASING AGE ON ANTISACCADIC EYE MOVEMENTS A. Olincy*, R.G. Ross, D.A. Young and R. Freedman. Schizophrenia Research Center, Univ. of Colorado Health Sci. Center, Denver, CO 80262.

As the brain ages, a decrease in frontal lobe volume with decreased cortical thickness, shrinkage of large neurons, cell loss and decreased density of synapses has been observed. Studies of aging in humans support decreased cerebral blood flow and metabolism in the frontal lobe. The antisaccadic eye movement task is known to be impaired in conditions with frontal lobe path logy. The antisaccadic task activates the frontal eye fields, supplementary motor area, thalamus, putamen and superior parietal lobe. This task measures motor, inhibitory and working memory abilities, functions that may become impaired with aging. Antisaccadic task performance decreases after the age of 60, with increasing numbers of prosaccades and increased latency of the antisaccade. There is no decrease in accuracy of the antisaccade once initiated. By examining the components of the antisaccadic task, impairment in frontal lobe functioning can be clarified.

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492.17

AGE-RELATED CHANGES IN THE RHESUS MONKEY: RECOGNITION MEMORY IN THE OLDEST OF THE OLD. M.B. Moss*, R.J. Killiany, D.L. Rosene and J. Herndon. Dept. of Anatomy and Neurobiology, Boston University School of Medicine, Boston, MA 02118 and Yerkes Regional Primate Research Center, Emory University, Atlanta, GA. 30322.

Assessment of recognition memory was performed in a group of five rhesus monkeys 29 to 31 years of age. Based on recently established life span data of rhesus monkeys, this age range represents the "oldest of the old". Their performance was compared to that of five young adult shimals (5 to 11 years of age) on two tasks of recognition memory: trial-unique delayed nonmatching to sample (DNMS), and delayed recognition span (DRST). The DNMS is a visual recognition task that requires the recognition of a novel from a familiar stimulus over a delay. The task was first administered with a 10 second interval between presentation of the sample and the recognition trial. Upon reaching learning criterion, performance was then assessed on two delay conditions with the interval between sample and recognition increased to 120 sec. and then 600 sec. The DRST is a recognition test that requires identification of a novel stimulus added to the increasing array of stimuli. The number of correct responses before committing an error constitutes the recognition span for that stimulus class. Animals were tested on two stimulus conditions, one using spatial position of identical plaques and the other using unique objects. Both a non-repeating and a repeating series of spans were used for each of the two conditions. The oldest of the old group was impaired on all conditions of the two tasks relative the young group. Relative to previous findings in a younger aged group (25-28) of monkeys, the oldest old were more impaired on acquisition of the DNMS task, but were not significantly different from this age range on other performance measures. The results point to a pattern of cognitive dysfunction toward the end of the life span that may be characterized by recognition memory impairment and a disproportionate decline in new learning.

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492.18

AGE-RELATED CHANGES IN THE RHESUS MONKEY: PATTERN OF COGNITIVE DECLINE. J. G. Herndon*, M. B. Moss, D. L Rosen and R. J. Killiany. Yerkes Regional Primate Res. Ctr., Emory Univ Atlanta, GA 30322 and Dept. of Anatomy and Neurobiology, Bosto Univ. Sch. of Med., Boston, MA 02118.

We evaluated the performance of young rhesus monkeys (<15 yea of age) on six measures of cognitive function to determine the prevalenc of impairment among early aged (19-23 years), advanced aged (24-2 years) and oldest old (29-33 years) monkeys. Impairment was define as performance that fell outside the range for that of young monkey: For early aged monkeys, prevalence rates were: (1) 25% (95% Confiden Interval - CI: 10-38%) on initial acquisition of Delayed Non-Matchin to-Sample (DNMS), (2) 42% (95% CI: 21-73%) on performance of t DNMS, with 2-min delay, (3) 67% (CI: 43-91%) on the spatial conditic of the Delayed Recognition Span Test (DRST), a test of short-ter spatial memory, (4) 0% (CI: 0- 31%) for memory span in a non-spati condition of the DRST, (5) 45 (CI: 24-77%) for in Spatial Reversal, test in which a positional preference must be reversed, and (6) 27% (C 11-61%) for Object Reversal. For acquisition f DNMS, DRST - N Spatial and Spatial Reversal, we detected a significant trend towa increasing prevalence of impairment in increasingly older groups of ag monkeys. Finally we used principal components analysis to derive Cognitive Performance Index (CPI), a measure of global cognitiv performance. The results confirm the multidimensional nature of ag related decline and provide the CPI as a measure representing the glob cognitive capacity of the individual. The CPI was negatively correlate with age ($r=-.78$, $n=53$, $p<0.001$). (Supported by NIH grants PO AG00001, R55 AG12610 and RR 00165).

Brain Damage by AIDS Under Active Study

Researchers agree that the AIDS virus injures the nervous system, but whether damage results from direct viral infection of brain cells or by indirect means is subject to controversy

SINCE 1985, when several groups of investigators reported that the AIDS virus not only enters the cerebrospinal fluid that bathes the brain and spinal cord, but also enters the brain itself, researchers have mounted a massive effort to determine how the AIDS virus so dramatically impairs nervous system function. Within the past year they have documented the neurological symptoms that many AIDS patients develop, ranging from mild confusion and poor coordination to profound dementia and an inability to control movement. Other scientists have identified the cell types—primarily macrophages and monocytes—that contain most of the detectable virus in the brains of AIDS patients. But even with this information in hand, the mechanisms by which the AIDS virus damage the nervous system have remained an enigma.

Now, new information is leading researchers to debate whether AIDS-related neurological damage is caused by direct viral infection of cells in the nervous system—glial cells in particular—or whether the virus mediates its damage indirectly, perhaps by inhibiting the actions of substances that are important for the survival or maintenance of the nervous system. Both ideas have some support, but the new evidence also calls into question the role of the T4 antigen, which on T lymphocytes binds the outer protein of the AIDS virus.

An emerging concept, new in terms of AIDS research but not new to many neuroscientists and immunologists, is that membrane proteins and responses to secreted factors that are shared by the nervous and immune systems may underlie much of the damage in AIDS. Perhaps because some of its proteins mimic the structure of naturally occurring substances, the AIDS virus may damage cells of the nervous and immune systems both directly and indirectly.

"Eventually, one-half to two-thirds of the 14,000 living AIDS patients in the United States will develop moderate to severe neurological problems." says Richard Price of the Memorial Sloan-Kettering Cancer Center in New York. Last year, Price, Bradford Navia, Carol Petito, and Enn-Sook Cho, also of Sloan-Kettering, reported that the evidence of damage to nervous system tissue, even in AIDS patients with severe symptoms, can be surprisingly subtle. Some, but not all, patients show a slight brain shrinkage, enlarged ventricles, abnormally staining white matter, or vacuolar myelopathy, a spinal cord abnormality in which the myelin sheaths surrounding nerve fibers contain abnormal spaces or vacuoles.

At about the same time, Anthony Fauci and Scott Koenig of the National Institute of Allergy and Infectious Diseases and their colleagues reported that 95% of the detectable virus in brain occurs in immune system cells, monocytes, or macrophages, some of which fuse to form multinucleate giant cells. Clayton Wiley, of the University of California at San Diego, and Jay Nelson and Michael Oldstone, of Scripps Clinic and Research Foundation in La Jolla, reported that some brain endothelial cells, which line blood capillaries, also contain virus.

To address the issue of how the AIDS virus severely impairs nervous system function, apparently without causing extensive structural damage to brain tissue, scientists are currently pursuing two major research strategies. One is to determine conclusively whether certain cells of the nervous system are susceptible to direct infection by the AIDS virus, and the other is to explore ways in which the virus may damage the nervous system indirectly.

Deborah Barnes

Richard Price *says that, "eventually, one-half to two-thirds of the 14,000 living AIDS patients in the United States will develop moderate to severe neurological problems."*

Several laboratories are now pursuing the first research strategy by determining whether neurons or glia grown in tissue culture or those in the brain of an AIDS patient can be infected with the AIDS virus. Jay Levy, Cecilia Cheng-Mayer, James Rutka, Mark Rosenblum, Thomas McHugh, and Daniel Stites of the University of California at San Francisco have preliminary evidence that the AIDS virus will infect two kinds of brain-derived cells in vitro. These are glioma cell lines, which are transformed glial cells—non-neuronal cells whose complex functions are not completely understood—and human fetal brain cells that have been subcultured several times.

"Three of the four glioma lines seem to replicate some viral isolates but not others," says Levy. "And the cultures that contain cells which stain positive for GFAP [glial fibrillary acidic protein], a marker for astrocytes, tend to be infected more often." The San Francisco group also finds that the source of a particular viral isolate does not predict the kinds of cells it will infect in vitro. Brain isolates infect some, but not all, cultures from brain and peripheral blood, and peripheral blood isolates show a similar heterogeneous pattern of infectivity.

Levy and his co-workers increase their ability to detect viral infection by adding peripheral mononuclear cells to the glioma and brain cell cultures, which Levy notes contain a variety of cell types. But even under enhanced conditions, the AIDS virus reproduces in these brain-derived cells at only 1/1,000 to 1/100,000 its level of replication in T4 lymphocytes.

Despite this low level of infection, however, Levy says that their data "clearly show that the AIDS virus will infect brain astrocytes in addition to infecting brain macrophages and endothelial cells. And cells do not need to have CD4 antigen [also known as the T4 receptor on T lymphocytes to which the AIDS virus binds] to be infectible with the virus." Levy, Nelson, and Wiley also have evidence that spinal cord oligodendrocytes, which make the fatty myelin sheaths that surround nerve cell axons, may also be infectible, possibly accounting for vacuolar myelopathy in some patients.

But David Ho of the UCLA School of Medicine is skeptical about in vitro evidence that the AIDS virus infects brain-specific cells directly. "The bulk of the evidence suggests that there is very little direct infection of neural cells," he says. "You can take

virus and add it to glial cell cultures, and if you also add T cells you can rescue the v[illegible] But you really don't know that the [illegible] entered the glial cells. It may have only stuck to the surface."

The notion that the AIDS virus actually infects glial cells in vivo as well as in vitro is strongly supported by new data from Joseph Melnick, Ferenc Gyorkey, and Phyllis Gyorkey of Baylor College of Medicine in Houston. They find that the AIDS virus infects two kinds of glial cells, both oligodendrocytes and astrocytes, in fresh brain biopsy tissue from AIDS patients. The researchers examined tissue from the neocortex of seven patients and found mature virus particles and staining for core protein of the AIDS virus in five of them. They indicate that mature and replicating virions, budding from infected cells, are generally rare, but occur more often in oligodendrocytes than in astrocytes.

"There is now no question that the AIDS virus is harbored in the brain," says Melnick. "What we see in our tiny samples of tissue probably represents what takes place in thousands of places in the brain of an AIDS patient." Melnick describes the Baylor group's recent efforts to find areas of brain cells that contained virus particles as "extremely laborious." He thinks that their ability to examine fresh, rather than postmortem, tissue was a key factor in making electron micrographs that show both intact virus and viral budding from brain glial cells.

Other laboratories are also trying to obtain convincing evidence that the AIDS virus infects brain cells directly in vitro, but with ambiguous results. For example, Suzanne Gartner of the National Cancer Institute (NCI) says, "We have not been able to get what I consider a productive infection in primary cultures of brain cells."

Gartner, Mikulas Popovic Elizabeth Read-Connole, and Robert Gallo, also of NCI, and Werner Mellert of the Institute for Biology Environmental Research Center in Munich, West Germany, are now trying "to see if we can mimic what happens in vivo," says Gartner. "In an AIDS patient, the cell type responsible for persistent brain infection is probably the macrophage. Infected macrophages are not like infected T cells; they don't die quickly, so they can continue to produce virus for a long time." To test how infected cells from the immune system might alter normal brain cells, Gartner and her co-workers add already infected T cells or macrophages to cultures of uninfected glioma and human fetal brain cells. "We have some preliminary evidence of infection, but only in a small number of cells," she says.

Their results are very preliminary, but the

Candidate AIDS Vaccine

"I do not have a vaccine for AIDS; I have a candidate vaccine," says Daniel Zagury of the Pierre and Marie Curie University in Paris, who has inoculated himself and ten Zairian volunteers with recombinant vaccinia virus containing a gene from the AIDS virus. News of the procedure elicited mixed reactions from U.S. scientists, some of whom expressed concerns about using the vaccinia virus. Zagury and his colleagues reported their findings in the 19 March issue of *Nature*.

"No complications up to now have occurred," said Zagury in a telephone interview with *Science*. Each of the people who received the vaccinia-based vaccine was initially free of antibodies to the AIDS virus, an indication that none was already infected. Zagury emphasized that his goal in the experiment was to "trigger signals that promote cell-mediated immunity" that would protect a person from different subtypes of the AIDS virus.

Vaccinia virus has been widely used to immunize people against smallpox and Zagury is testing a genetically engineered form of it that contains a gene from the HTLV-III_B isolate of the AIDS virus that codes for gp160, the envelope glycoprotein composed of gp120 and gp41. Zagury's primary immune response was to make antibodies that killed the same viral subtype in vitro, but failed to neutralize HTLV-III_{RF}, a Haitian isolate that differs genetically. The vaccine also stimulated two cell-mediated responses—lymphocyte mitosis and expression of T-cell receptors for interleukin-2—when Zagury's lymphocytes were exposed to either viral isolate in vitro.

Zagury received the biological materials necessary to produce the candidate vaccine as a result of his prior collaboration with Robert Gallo and Marjorie Robert-Guroff of the National Cancer Institute (NCI), and Bernard Moss of the National Institute of Allergy and Infectious Diseases (NIAID).

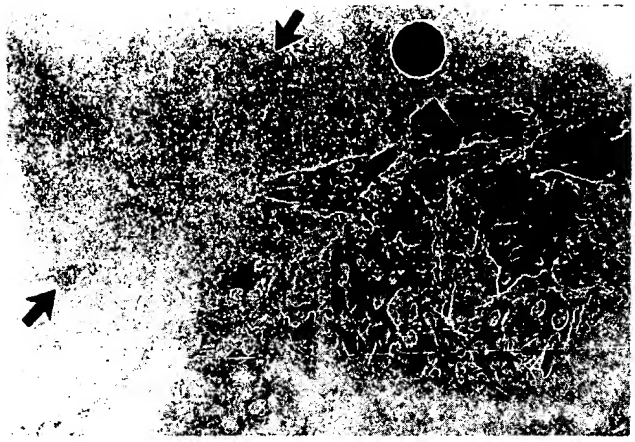
Moss sent Zagury two kinds of materials that were intended for experiments in animals, not in humans. One was a recombinant vaccinia virus that expresses the AIDS gp160 protein and the second was a plasmid preparation (which contained small circular chains of viral DNA that included the AIDS virus gene) from which Zagury could make other recombinant viruses. Moss was surprised to learn in December that Zagury had injected himself with the engineered vaccinia virus.

"This was not a collaboration on my part; Zagury simply used material intended for African Green monkeys," says Moss. The prototype vaccine has not been reviewed or approved by the Food and Drug Administration (FDA) for use in humans. It is based on the WR laboratory strain of vaccinia not normally used in humans because it is not produced under stringent laboratory conditions. Although Zagury initially used the WR vaccinia strain in his candidate vaccine, he indicates that new preparations of the vaccine are based on the Lister strain of vaccinia, which has been used in humans.

Zagury says that animal testing done in collaboration with the NCI and NIAID researchers demonstrated that the vaccine procedure was innocuous. The new work "has the full support of the Zairian ethics committee," he reports.

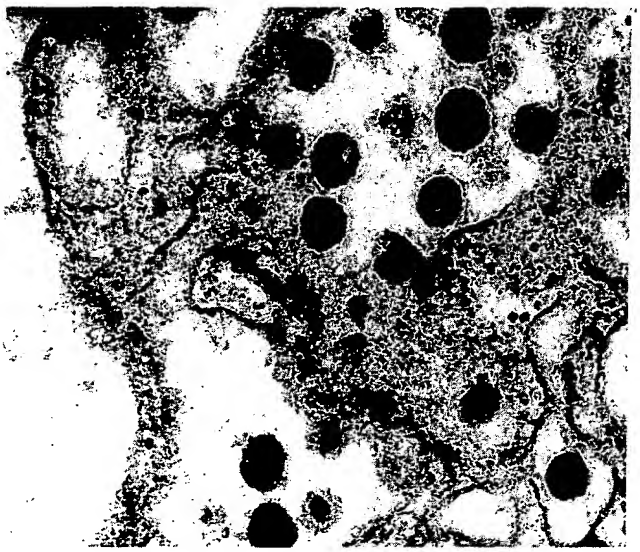
Anthony Fauci of NIAID describes the candidate vaccine trial as "potentially a very exciting study." He says that this limited study begins to address the concern that such a vaccine might induce death of cells carrying the T4 antigen. Researchers have worried that gp120 might cause T4 lymphocytes, which bind the protein, to fuse together, thus reducing their number and decreasing a person's ability to mount cell-mediated responses. This did not seem to occur according to Zagury's preliminary results, however. Fauci also notes that Zagury's findings do not say if cell-mediated cytotoxicity was induced. This form of immunity may play a very important role in eliminating cells in the body that are infected with the AIDS virus.

Zagury says that he also treated ten AIDS patients to try to induce cell-mediated immunity, but used an entirely different experimental protocol. Two patients in July and eight more in December "received AIDS virus-infected cells that had been fixed with formaldehyde," Zagury says. The formaldehyde rendered the cells noninfectious but preserved their ability to stimulate an immune response, according to Zagury. He did not comment further on the outcome of these trials. ■ **D. M. B.**



AIDS in vitro

The AIDS virus infects some transformed glial cells in culture, which stain darkly because they are producing viral protein. "Uninfected cells" (or cells not expressing viral protein) can be seen in the background (arrowheads). [Photo courtesy of Werner Mellert]



AIDS in vivo

An AIDS virus–infected glial cell in the brain of a patient actively produces virus particles, some of which have bar-shaped cores. The infected cell is identified as an oligodendrocyte because it is producing myelin (not seen at this high magnification; ×85,000). [Photo courtesy of Joseph Melnick]

NCI group is finding that infected macrophages do alter noninfected glioma cells, and they propose two mechanisms by which this might occur. One is that the virus infects brain cells directly. The other is that macrophages, which secrete regulatory molecules under normal circumstances, secrete abnormal factors when they are infected by the AIDS virus. "I think the viral infection is changing the macrophage and then its function is changed," Gartner says.

Not all researchers are convinced that brain macrophages play a primary role in nervous system damage, however. "Everyone is euphoric, thinking that macrophages bring virus into the brain," Price says. "But macrophages could just be an indicator of infection. Suppose a very low level of virus infects the brain. Then, the macrophages could come in and pick up virus and amplify the infection. There is no evidence that this happens, but there is also no evidence that macrophages bring virus into the brain either."

Price also questions why it is often difficult to find evidence of viral infection in patients with neurological symptoms. "We find virus in the brain of only about one-third of the demented patients," says Price. "An obvious explanation is that the detection techniques are not sensitive enough. But one of the questions that people have raised is, 'Are there some indirect methods by which the virus damages the nervous system?' "

Scientists who pursue this second major research direction envision several possibilities by which damage may occur. First, as Gartner suggests, infected cells may secrete altered factors that can no longer perform some necessary function. Second, infected cells may secrete substances that are actually toxic to some cells. Or third, the AIDS virus may compete for receptor binding sites on cells of the nervous system that would normally be occupied by necessary maintenance or survival factors.

At least two different groups of investigators, Mark Gurney and his colleagues at the University of Chicago and Candace Pert and her co-workers at the National Institute of Mental Health (NIMH), have recently described data that point to the third possibility—specifically, that gp120 (the envelope glycoprotein that surrounds the AIDS virus) may mediate damage indirectly.

Last fall, Gurney, Mark Lee, and Brian Apatoff reported that neuroleukin, a novel factor secreted by stimulated T lymphocytes, promotes the survival of a population of embryonic chick sensory neurons that is insensitive to nerve growth factor. The researchers suggested that, because a region of the neuroleukin protein is partly homologous to gp120 and because neuroleukin can activate B lymphocytes to secrete immunoglobin, there may be a common mechanism by which a product of the AIDS virus both alters immune system function and interferes with neuronal function.

Now, Gurney, Lee, Apatoff, Gregory Spear, and Indre Rackauskas, also of the University of Chicago, in collaboration with Ho have preliminary evidence that fragments of the AIDS virus envelope glycoprotein inhibit the ability of neuroleukin, but not nerve growth factor, to enhance the survival and maintenance of certain nerve cells in vitro. Their new work also shows that brain neurons, specifically septal and hippocampal neurons, respond to neuroleukin, indicating that the trophic factor may work in the central, as well as the peripheral, nervous system.

As yet, however, Gurney and his coworkers have not been able to show that neurons have specific membrane receptors for neuroleukin or to determine whether the AIDS virus envelope glycoprotein competes with neuroleukin for specific binding sites on nerve cells. Additionally, the researchers do not know if adult neurons in the human brain require neuroleukin as a survival factor. But, says Gurney, "with neuroleukin, you have a growth factor that is highly homologous in different species—it affects human B lymphocytes, embryonic mouse brain neurons, and embryonic chick sensory neurons. It may be that a gene product of the AIDS virus, the envelope glycoprotein, is released by infected macrophages in the brain and that it inhibits the function of neuroleukin."

Pert and Joanna Hill, also of NIMH, and William Farrar of the Frederick Cancer Research Facility in Maryland, along with their colleagues also have evidence that the AIDS virus may interfere with the action of a normally occurring substance in the brain. They suggest that a peptide—which Pert and Michael Ruff, of the National Institute of Dental Research, now think is vasoactive intestinal peptide—binds to the T4-like antigen the group has recently identified in brain tissue from rats, squirrel monkeys, and humans. "It could be that parts of the brain—the hippocampus, dentate gyrus, amygdala, and outer layers of the cerebral

ortex in particular—are areas of attachm[illegible] for the AIDS virus," says Hill. "This co[illegible] mean that they are sites for infection."

But the accumulating evidence about where and how the AIDS virus affects the brain sometimes points in different directions. For instance, Price's group finds viral antigen staining in AIDS patients' brains, not at the cortical sites that contain T4 receptor, but in the gray matter underlying the cerebral cortex and in the white matter.

Two possible resolutions of this apparent contradiction are that the brain T4 antigen is not a binding site for the AIDS virus or that it is a site that allows binding but not infection. Pert and her colleagues favor the latter explanation and speculate that the AIDS virus may indirectly damage brain neurons important for intellectual function and emotions. "There is a possibility that the T4 antigen is acting as a receptor for an endogenous peptide," Hill says. "But when it is covered up with the AIDS virus, then the endogenous substance may not be able to bind." By this indirect mechanism, the AIDS virus could cause brain damage because it prevents the interaction of brain cells with a substance, perhaps vasoactive intestinal peptide or a related peptide, that is necessary for their survival or function.

Adding to the body of evidence that a T4 receptor is a component of brain tissue, Charles Gerfen and Paul St. John of NIMH have preliminary data suggesting that the T4 receptor "is located on brain neurons and not on glia." They find that staining for the T4 antigen occurs on cells that appear to be neurons in cultures of rat hippocampus. The cells with T4 antigen look like those that "have neuronal morphology and stain positive for neurofilament and tetanus toxin [two markers for nerve cells]," Gerfen says, although he and St. John have not yet demonstrated T4 staining and neuronal markers in the same cells.

Because much research effort is directed toward showing what brain cells have T4 antigen, a critical question is whether the presence of that receptor means that the cell displaying it is susceptible to infection and damage by the AIDS virus. Many researchers think that cells must have the antigen. But, because Levy and his co-workers find that several isolates of the AIDS virus can infect cells lacking detectable levels of T4 antigen, Levy does not think that the T4 receptor "is the sole factor underlying infectivity. Instead," he says, "something about the ability of a viral isolate to replicate makes it different and therefore affects its ability to damage different cells." He proposes that the crucial differences among viral isolates that make them more infectious lie in the core or the 3′ *orf* end of the genome, rather than in the envelope glycoprotein, which is known to interact with T4 receptors on lymphocytes.

Farrar suggests that the AIDS virus may infect cells by more than one mechanism and proposes two possible modes of entry. One is the result of binding to the T4 receptor, and the other is "probably not receptor-mediated," he says. "In general, all receptor-mediated processes will occur quickly. But a process that is more amorphous—I like to call it random binding—may occur more slowly. Virus could be incorporated into a cell as a part of the pinocytotic or cell-drinking process, if there is enough of it around." Whether cell types, such as neurons, that actively recycle membrane at their synaptic terminals, take up virus by this method has yet to be determined.

Farrar also thinks that certain products of the AIDS virus genome may, in themselves, be damaging to cells, and he leans toward gp120, the envelope glycoprotein that surrounds the AIDS virus, as the culprit. "We find that purified gp120 by itself has physiological consequences," he says. Although Farrar and Douglas Ferris, also of Frederick, have yet to test the effects of gp120 on neural tissue, his hypothesis adds another possible dimension to the kind of damage the AIDS virus may do to the nervous system—specifically, that direct damage mediated by viral proteins, rather than by the intact virus, might occur.

Still other research points to the idea that much nervous system damage in an AIDS patient is due to infections by different agents, particularly cytomegalovirus (CMV). Because the AIDS virus attacks T4 lymphocytes and suppresses immune system function, many patients with neurological problems are susceptible to multiple brain infections. Wiley proposes that, by infecting the brain of an AIDS patient, CMV can elicit an immune response which then may draw AIDS virus–infected mononuclear cells into the brain.

"Sixty-seven percent of our patients have CMV infections," says Wiley. He and his colleagues are reexamining brain tissue from AIDS patients collected over the past 5 years and find that, for some unexplained reason, an increasing percentage of the brain samples have cytomegalovirus infection and damage. Although CMV brain infections are not common, they arise frequently in AIDS patients, and the DNA herpes-like virus infects both neurons and glia, he says.

Until recently, many researchers were pessimistic that AIDS-related damage to the nervous system could be reversed. But within the past year, Samuel Broder of NCI and his colleagues and Dannie King of Burroughs Wellcome in Research Triangle [illegible]k, North Carolina, have reported that 3′-[illegible]ido-3′-deoxythymidine (AZT) appears to slow, or even reverse, some of the neurological symptoms in a subset of AIDS patients (see *Science*, 20 March, p. 1462).

Broder and his co-workers report that six of seven patients with neurological symptoms who received AZT improved in intellectual or peripheral nerve function. Some improved only temporarily, probably because their doses of AZT had to be lowered, and Broder is cautious about overinterpreting the preliminary results. "These data should not be taken as a final answer, but as an encouragement to do the necessary large-scale studies. Then we may be able define what kinds of neurological improvements we can expect."

Researchers still do not know precisely how the AIDS virus damages the nervous system, but within the past year they have proposed several hypotheses and are actively testing their ideas. The intact AIDS virus or its protein products may injure the nervous system directly, damage it indirectly, induce nonneural cells to secrete substances that are toxic to neural tissue, or act through a combination of mechanisms. As scientists learn more about how the virus affects cells of the immune system, they may find that many of the same mechanisms are responsible for nervous system damage. ■

DEBORAH M. BARNES

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Neurotrophic Factors Enter the Clinic

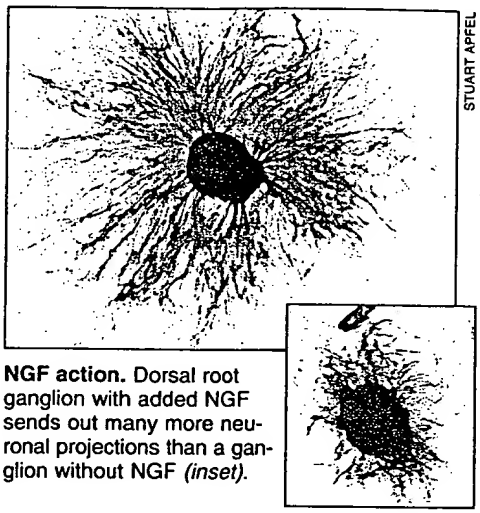
The biotech industry launches a new class of nerve-nurturing drugs with high hopes of toppling stubborn neurological diseases such as Lou Gehrig's disease

Neurons are among our most precious cells. They play vital roles in our lives, governing everything from the recoil of a finger from a hot stove to the understanding of Dante. Unlike the vast majority of other cells, however, most neurons must perform these all-important tasks for an entire lifetime, as they aren't replaced if destroyed by injury or disease. Given that neurons are so precious, it's not surprising that one of the hottest areas in neuroscience today is the study of neurotrophic factors: naturally occurring proteins that keep neurons alive and healthy during embryonic development and later in normal adult life.

This interest in neurotrophic factors is not merely academic. In fact, it has already generated a "wave of excitement" in the biotech world, says Jeff Vaught, senior vice president for research at Cephalon, a biotech company in West Chester, Pennsylvania. A half-dozen or more companies have clinical trials planned or under way for testing neurotrophic factors against debilitating neurodegenerative diseases, including Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease.

"Most of the diseases for which these neurotrophic factors are being developed are diseases for which current therapies are virtually nonexistent," says biotech stock analyst Margaret McGeorge of Hancock Institutional Equity Services in San Francisco. That lack of treatments, she says, is a driving force behind the current enthusiasm. But McGeorge also points out that "it is much too early to say" which factors will pan out as useful drugs. One highly touted neurotrophic factor has already stumbled in clinical trials—possibly a result of being rushed into large-scale trials. That experience hints that bringing these nerve-nurturing drugs to market could pose bigger challenges than companies originally thought. Still, that setback has only slightly dimmed the excitement that surrounds neurotrophic factors in the research and biotech communities.

That excitement has been building to its current crescendo for a long time, beginning more than 30 years ago with the Nobel prize–winning discovery of the first neurotrophic factor—nerve growth factor (NGF)—by Rita Levi-Montalcini, Stanley Cohen, and Viktor Hamburger at Washington University in St. Louis. They showed that NGF ensures the survival of certain peripheral neurons as they grow toward and connect with target tissues during the development of the nervous system. Later work showed that NGF is needed for the survival of some brain neurons as well.



STUART APFEL

NGF action. Dorsal root ganglion with added NGF sends out many more neuronal projections than a ganglion without NGF *(inset)*.

But many types of nerve cells didn't respond to NGF, raising the possibility that they are supported by other factors. The search for those factors has so far uncovered roughly a dozen proteins that help neurons survive throughout the body and brain. Some, like NGF, work only on some types of nerve cells. Others, such as insulin-like growth factor (IGF) and fibroblast growth factor (FGF), were originally identified as growth factors for other tissues, such as muscle, or the immune system, and were only later found to nurture neurons as well. As the field has grown, the definition of a neurotrophic factor "has been expanded a lot," says Washington University neuroscientist Eugene Johnson, "and it's clear that there are a lot more players."

ALS: A cautionary tale

As this cast of characters expanded, researchers quickly realized that some of the newly discovered growth factors might serve as therapeutic agents for neurological diseases. The first disease to be targeted by neurotrophic factors in a major clinical trial was ALS, a deadly condition in which the motor neurons—neurons that control the skeletal muscles—progressively degenerate, eventually depriving patients of all movements, even the ability to breathe.

ALS was a good starting point for several reasons. For one thing, there's no effective treatment for the disorder. In addition, motor neurons, unlike neurons of the brain, are accessible to protein drugs introduced into the bloodstream. Says Cephalon's Vaught, diseases like ALS should be "very amenable to growth-factor application because the projections from the affected neurons lie outside the blood-brain barrier," the physiological wall that keeps large molecules like proteins from entering the brain.

A number of growth factors had already been shown to support the growth of motor neurons in laboratory cultures, and three of those factors became early candidates for treating ALS: brain-derived neurotrophic factor (BDNF) and insulin-like growth factor I (IGF-I), both made by muscles controlled by motor neurons, and ciliary neurotrophic factor (CNTF), made by the Schwann cells that form an insulating sheath around the neurons.

In addition to the cell-culture work, all three factors also turned out to aid the healing of injured motor neurons in rats. Moreover, the factors improved the condition of mice with hereditary motor-neuron diseases similar to ALS. That was enough for two companies, Regeneron Pharmaceuticals of Tarrytown, New York, and Synergen of Boulder, Colorado, to begin clinical trials of CNTF and for a third, Cephalon, to begin trials with IGF-I. Regeneron is also developing BDNF, in collaboration with Amgen of Thousand Oaks, California, although they have not yet begun large-scale trials.

But the high hopes with which these trials began quickly turned into what many observers view as a cautionary lesson for the field. The first publicly reported results with CNTF looked promising enough: Last September, at the World Congress of Neurology in Vancouver, British Columbia, Regeneron announced that 12 ALS patients who had been receiving CNTF injections in a small, preliminary trial showed less of a decline in muscle strength than did 14 patients receiving a placebo. The results were not statistically significant, Jesse Cedarbaum, vice president for clinical affairs at Regeneron, told *Science* in an interview last November, but they were sufficiently encouraging to justify a full-scale trial of CNTF's effectiveness. Last year Regeneron began that trial, which includes 720 patients at 36 clinical sites.

But even as Regeneron trumpeted its ear-

ly results, optimism about CNTF had already begun to erode. Rumors were circulating that patients in a preliminary safety trial of CNTF carried out by Synergen had suffered side effects, including coughing, fever, weight loss, and activation of herpes virus, which can hide in latent form in some neurons.

Similar side effects were also rumored to be plaguing Regeneron's large-scale trial—rumors that were soon to be borne out. In March, Regeneron researchers announced that they had unblinded the data on 550 patients who had completed 6 months of the trial and found that a substantial number of those receiving CNTF had not only had serious side effects, but had actually fared worse on measures of muscle strength than did patients receiving placebos. Patients who didn't experience side effects showed modest improvement as compared to controls, but investors weren't mollified by that scrap of good news, and Regeneron's stock dropped 50%. The company has tried to salvage the trial by applying to the Food and Drug Administration to continue it with lower doses aimed at reducing the side effects.

But the question remains whether anything more than modest effects can be expected of CNTF. Synergen has a large trial in progress, using lower drug doses than Regeneron, based on the findings of its safety trials. The trial has not been unblinded, but insiders say the prospects don't look very exciting. "Although all the numbers haven't been added up [for either large-scale trial], the clinical results in ALS are less than we had hoped for," says Tufts University neurologist Theodore Munsat, who has participated in the Synergen trials. The best that can be expected, says Munsat, is "a modest slowing of the deterioration rate." He questions whether such modest effects would improve the quality of a patient's life enough to justify use of the drug.

But some researchers think the first round of trials is not a fair test of CNTF. Neurologist Michael Sendtner of the Max Planck Institute for Psychiatry in Martinsried, Germany, argues that the way the drug is administered could be key to reducing its side effects and improving its effectiveness. He recently found, for example, that CNTF has a half-life of only 3 minutes in the bloodstream of a rat. That means the injected drug has little time to reach the motor neurons. While in the bloodstream, it encounters tissues such as the liver and lungs and also interacts with blood-borne immune cells. CNTF's interaction with these cells and tissues, which don't usually encounter the factor, may account for its side effects, says Sendtner. He suggests there is still hope for better results if CNTF were delivered directly to the cerebrospinal fluid, which bathes the spinal roots of the motor neurons.

Even if CNTF doesn't pan out, biotech researchers hope IGF-I will have fewer drawbacks as an ALS therapy. That drug has already been used in large-scale drug trials for conditions including diabetes, dwarfism, and osteoporosis without showing serious side effects. "The good news about IGF-I is that it certainly appears so far to be fairly benign," says biotech analyst Timothy Wilson of the New York securities firm Hambrecht & Quist. "But the big question mark is whether or not it will have efficacy in ALS." The Cephalon trial aims to answer that question.

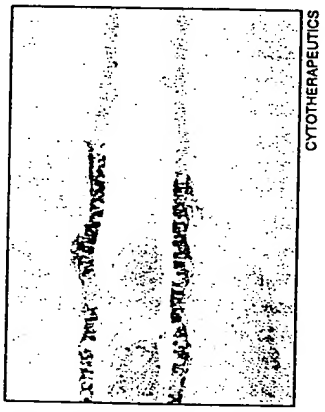
But even if other companies do hit pay dirt with an ALS treatment, close observers of the field think Regeneron's experience holds some lessons for all the companies involved. Some argue, for example, that Regeneron moved precipitously, relying on results from early trials that were too small for adequate analysis of optimal dosing. "When companies have tried to move too quickly through these clinical trials, they usually find disappointment," says R. Brandon Fradd, a biotech stock analyst with Montgomery Securities in San Francisco. But Fradd and others emphasize that, despite the first disappointing results, the field of neurotrophic factors still shows promise.

Peripheral neuropathy: Better odds

Some of that promise may be realized in tackling a more straightforward problem. One reason finding ALS therapies is so difficult is that the cause of the motor neuron degeneration remains unknown, so there is no good animal model for the disease. The story is different for peripheral neuropathies: the deterioration of sensory or motor neurons

Solving the Delivery Puzzle

Neurobiologists have high hopes that neurotrophic factors, naturally occurring proteins that help keep nerve cells healthy, may one day provide effective therapies for a wide variety of neurodegenerative diseases (see main text). But before that day comes, they will have to solve a problem: how to administer the factors safely and effectively. It's unlikely that the factors will ever be available as pills that can be washed down with a gulp of water, because they are proteins and thus would be rapidly destroyed in the digestive tract before they could be absorbed. For diseases of the peripheral nerves, the drugs may be injected into the patient's skin, but for brain conditions such as Alzheimer's or Parkinson's disease they may need to be pumped directly into the brain to circumvent the blood-brain barrier, which keeps proteins carried by the bloodstream from reaching the brain.



Growing in. GDNF in capsule attracts growing neurons.

But even though these approaches are being used in the early clinical trials, each has inherent disadvantages. Injecting a trophic factor into the skin sends it into the bloodstream, allowing it to influence many tissues, possibly causing unwanted side effects. And a catheter pumping a trophic factor directly into the brain could be a conduit for dangerous infections. That's why researchers are working to develop more sophisticated delivery systems.

One possibility, under development at CytoTherapeutics Inc. in Providence, Rhode Island, involves enclosing neurotrophic factor–producing cells in tiny, semipermeable capsules that can be implanted anywhere in the body. The capsules—which have been tested in animals and humans—can be implanted in the brain or in the cerebrospinal fluid at the base of the spine. They let nutrients and cellular products pass in and out, but they keep the cells contained and isolated from the recipients' immune defenses. "You can almost think of it as a biological pump," says biotech analyst Margaret McGeorge of Hancock Institutional Equity Services in San Francisco. "You have got a little factory in there, churning out [trophic factor]."

Another strategy is to engineer a person's own skin cells to produce a neurotrophic factor and then tranplant the engineered cells into the brain—a method being tested in rats and monkeys by Fred Gage at the University of California, San Diego. Or the protein factors might be joined with molecules that help them slip across the blood-brain barrier. That approach, being developed by scientists at Alkermes Inc. in Cambridge, Massachusetts, would allow trophic factors injected into the skin to still reach the brain. With these and other approaches in the pipeline, researchers have a rich supply of potential tools to get these nourishing factors to diseased neurons that need them to survive.

–M.B.

caused by diabetes or by treatment with cancer chemotherapeutic agents such as vincristine, cisplatin, and taxol. For those conditions, there are good models. "You can cause peripheral neuropathy with vincristine in a rat and in man," says Wilson, "and if IGF-I prevents it in the rat, there is a very good chance it will do so in man, because it's the same chemical insult."

And this condition has already been met, since IGF-I does prevent drug-induced peripheral neuropathies in animals, as does NGF. Jack Kessler, Stuart Apfel, and their colleagues at Albert Einstein College of Medicine in New York have collaborated with Cephalon and with Genentech of South San Francisco to show the effectiveness of these two factors against drug-induced neuropathies in mice.

As in the rest of this field, clinical trials are following very quickly behind laboratory results; Cephalon is planning to begin clinical trials of IGF-I for peripheral neuropathy within a few months. Genentech has already completed a small multicenter dosing and safety trial of NGF in human patients with peripheral sensory neuropathy, and expects to begin a larger trial by this summer, which would include diabetics with sensory neuropathy. They are tackling diabetes first, says Kessler, who is participating in the studies, because "diabetic neuropathy is a much more prevalent and disabling medical problem" than neuropathy from cancer chemotherapy. As a result of neuropathy, diabetics often experience ulceration of their feet and legs, which can eventually require amputation.

But those two neurotrophic factors aren't the only ones being tried out against neuropathy. Genentech scientist Gao Wei-Qiang recently demonstrated that a factor called neurotrophin-3 (NT-3) protects mice from neuropathy caused by the anti-cancer drug cisplatin, says Franz Hefti, director of Genentech's neuroscience department. The upshot, says Hefti, is that "for peripheral neuropathy a combination of neurotrophins is likely to do the trick."

Parkinson's and Alzheimer's: A fresh approach

Most of the clinical trials now underway have concentrated on testing neurotrophic factors as treatments for conditions such as ALS and peripheral neuropathy in which peripheral nerve cells deteriorate. But that kind of therapy doesn't exhaust the possible uses of the trophic factors. One other important area where these proteins are now beginning to play a role is as experimental therapies for neurodegenerative diseases of the brain.

Alzheimer's is the most prevalent of those diseases, and the leading candidate for Alzheimer's therapy is NGF. An early clue that NGF might be of benefit came from experiments on one of the major groups of neurons that degenerate in Alzheimer's—the cholinergic neurons. These neurons (so called because they release acetylcholine as a neurotransmitter) send their projections into a memory center in a brain structure called the hippocampus. Several research groups have shown that when cholinergic neurons in rat brains are damaged by having their projections cut, they can be saved from dying by infusions of NGF.

Further experiments suggest that saving those neurons may have consequences for mental function. When Fred Gage and coworkers at the University of California, San Diego, infused NGF into the brains of aging rats that showed memory impairment, they found what Gage describes as "very good improvement in [learning] behavior." On a test of memory retention, he adds, the NGF-treated rats "were in the range of the aged nonimpaired group."

Encouraging as these results are, it's far too soon to imagine that NGF will do the same thing for Alzheimer's patients that it did for aging rats. For one thing, the rats are not a perfect animal model for Alzheimer's. Furthermore, it isn't clear whether the decay of the cholinergic neurons is in fact a cause or an effect of human Alzheimer's. Nevertheless, the animal results suggest that keeping the cholinergic neurons alive can improve mental function, says Genentech's Hefti.

Given that possibility, Alzheimer's researchers are ready to see what effects the neurotrophic factor might have in human beings. Lars Olson and his colleagues at the Karolinska Institute in Stockholm have already tried NGF infusion in one Alzheimer's patient; they report that the patient showed improvement on a memory test. Both Genentech and Synergen, the latter in collaboration with Syntex, are planning clinical trials of NGF for Alzheimer's disease as soon as next year. "We don't expect NGF to cure the disease," says Hefti, "but we expect NGF to have a significant behavioral effect."

Researchers also have high hopes for neurotrophic factors in the treatment of Parkinson's disease, another common and intractable neurodegenerative disease, which is characterized by the degeneration of certain dopamine-producing neurons in the brain. Last year, Frank Collins and his colleagues at Synergen purified a protein they call glial cell-line derived neurotrophic factor (GDNF), which supports the survival, in cell culture, of the neurons that die in Parkinson's disease. Using GDNF, "we are hoping that we can reverse the disease," says Synergen chief executive officer Larry Soll. GDNF won't bring a dead neuron back to life, he notes, but it might do something almost as good: Experiments in rats and mice by Synergen researchers suggest GDNF can coax surviving neurons to send out new projections to replace those from lost neurons. If it has similar effects in primates, Soll says Synergen hopes to have GDNF in clinical trials by 1995.

Although the future of any single therapeutic approach with neurotrophic factors remains uncertain, the possibilities are numerous enough to fuel several generations of clinical trials, using increasingly sophisticated delivery systems (see box). And even if the first round comes up short of expectations, observers say it is only a matter of time until our besieged neurons find new pharmaceutical allies in the form of proteins designed by nature to keep neurons alive.

–Marcia Barinaga

Some Neurotrophic Factors Moving Toward Clinical Trials

Factor	Diseases	Companies developing
Nerve growth factor (NGF)	Peripheral neuropathy Alzheimer's disease Spinal-cord injury	Genentech Synergen/Syntex
Brain-derived neurotrophic factor (BDNF)	Amyotrophic lateral sclerosis (ALS) Parkinson's disease	Amgen/Regeneron
Neurotrophin-3 (NT-3)	Peripheral neuropathy	Genentech Amgen/Regeneron Takeda
Ciliary neurotrophic factor (CNTF)	ALS Motor neuron diseases	Regeneron Synergen
Glial cell-line derived neurotrophic factor (GDNF)	Parkinson's disease	Synergen
Insulin-like growth factor I (IGF-I)	ALS Peripheral neuropathy Motor neuron diseases Post-polio syndrome	Cephalon

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Neurotrophic factors: ready to go?

Nerve growth factor (NGF), the prototype neurotrophic factor, was found several decades ago[1]. It has been shown to promote growth, survival and differentiation of neurons[2–4], and its potential therapeutic use was obvious early on. However, progress in this area has been slow. Within the past few years, a variety of similar factors and their corresponding receptors have been characterized[2,4,5]. These include members of the classical neurotrophin family such as brain-derived growth factor (BDNF), neurotrophin 3 (NT-3), NT-4/5, the ciliary neurotrophic factor (CNTF), members of the fibroblast growth factor (FGF) family, insulin-like growth factor I (IGF-1), transforming growth factor β (TGF-β) and glial-derived growth factor (GDNF), which has been characterized very recently.

Many studies using cell-culture and animal models have shown that neurotrophic growth factors are able to prevent or inhibit neuronal death induced by a variety of insults[6–9]. Can these results be transferred easily to humans, and will they provide sufficient preclinical data for starting clinical trials[10]? A clinical trial using CNTF was unsuccessful because of unexpected side-effects. Therefore, it is important to choose the right factors for the most appropriate patient group with, for example, the correct dosing schemes to demonstrate the clinical feasibility of these molecules in human diseases. The potential use of neurotrophic growth factors as drugs for the treatment of neurological and sensory disorders was the topic of a meeting earlier this year*.

Disorders and neurotrophic factors

Theoretical indications for the beneficial use of growth factors exist for neurodegenerative diseases, brain injuries and peripheral neuropathies resulting from several etiologies. Neurodegenerative diseases are highly devastating disorders, and a characteristic feature of these diseases is the damage and loss of certain neuronal populations[6,7,11]. The ultimate goal would be to protect and rescue these neurons, restore proper function and prevent further progression.

Several neurotrophic factors have been implicated in survival and differentiation of spinal motor neurons[8,9]. Insulin-like growth factor I is effective in various animal models of motor-neuron disorders and no adverse side-effects have been observed in ongoing clinical trials (Jeffry Vaught, West Chester, PA, USA). There is systemic bioactivity allowing subcutaneous application. In the animal models, CNTF or BDNF alone has a moderate effect, but the effects of BDNF and NT-3 are additive, and the effects of BDNF and CNTF are synergistic (Ronald Lindsay, Tarrytown, NY, USA).

One pathological characteristic of Alzheimer's disease (AD) is neuronal loss resulting in deficits in three neuronal systems: cholinergic, hippocampal and cortical neurons[6,7]. Nerve growth factor showed a robust protective effect in the cholinergic system in cell-culture and animal models[6]. This role was reinforced in an elegant study in transgenic mice lacking NGF, where NGF was reintroduced by peripheral expression (Franz Hefti, South San Francisco, CA, USA).

The pathological characteristic of Parkinson's disease (PD) is the loss of dopaminergic neurons in the substantia nigra. Klaus Unsicker (Heidelberg, Germany) discussed the application of FGF-2 and TGF-β and of chromaffin-cell transplants in various models[11]. A particular problem in PD is the difficult nature of the application of growth factors, and, therefore, clinical trials in PD are not yet imminent. Interestingly, in a recent issue of *Nature*, there were four articles addressing the role of GDNF in animal models for PD (Refs 12–15).

Peripheral neuropathies are common and are often debilitating. They involve various neuronal populations, depending upon the particular etiology[16]. Some anticancer drugs (for example, Taxol, vincristine and cisplatin) and diabetes can cause these diseases. By contrast with neurodegenerative diseases, peripheral neuropathies are easier to study because reliable animal models exist for them, they are more accessible allowing systemic drug application, and the character of the disorders is more straightforward. Once again, the goal is to create a more normal phenotype, arrest the disease and stop the progression.

A variety of neurotrophic factors was studied for their effects on sensory deficits resulting from degeneration of sensory receptors or the nerves innervating them. An ideal aim would be to regrow lost receptors. There is a huge demand for treating deficits of the eye and ear, and accordingly, the visual and auditory systems were discussed to a greater extent than the olfactory system. Although remarkable insight into the pathogenesis of these disorders is emerging rapidly and considerable progress has been made, it was obvious that neurotrophic factors as drugs are still far from re-establishing lost function.

The neurotrophic factors that might be effective in particular diseases are shown in Table I (for reviews, see Refs 1–4 and 10). Eventual combination therapies might be difficult to realize since companies are focusing on drugs where they hold the patent rights. However, companies are screening extensively for altered molecules with improved bioavailability, novel factors or receptors. Hefti pointed out that NGF alone might only improve the damage in cholinergic neurons in AD, and hence more than a partial effect of NGF in clinical trials would be surprising. Additional components that target hippocampal and cortical neurons are required to revert the disease fully. One promising factor–receptor combination has been identified by using a screening strategy for novel tyrosine-kinase receptors in the cortex and then characterizing the ligands.

TABLE I. Ongoing or initiated clinical trials

Neurotrophic factor	Disease
Nerve growth factor	Diabetic neuropathy Taxol neuropathy Compressive neuropathy AIDS-related neuropathy Alzheimer's disease (some patients in Sweden)
Brain-derived growth factor	Amyotrophic lateral sclerosis
Neurotrophin 3	Large fiber neuropathy
Insulin-like growth factor	Amyotrophic lateral sclerosis Vincristine neuropathy Taxol neuropathy
Ciliary neurotrophic factor	Amyotrophic lateral sclerosis

Growth Factors as Drugs for Neurological and Sensory Disorders. Held in London, UK: 25–27 April 1995.

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Box 1. Possible delivery systems for neurotrophic factors

Systemically (only some disorders)
Intra-cerebroventricular (ICV) pump system
Intraparenchymal injection
Installation of polymers with slow release
'Ex vivo' gene therapy:
  grafting of genetically altered cells
  encapsulated cells (immuno-isolation)

Future applications:
  'in vivo' gene therapy (Adenovirus- or Herpes virus-based vectors)
  molecules crossing blood–brain barrier
  active peptides, pantrophic molecules,
  drugs with indirect effects on receptors

Experimental models

The pathological features of amyotrophic lateral sclerosis (ALS) are composed of cytoskeleton and neurofilament structures, followed by axonal swelling with inclusions and apoptotic cell death. Donald Price (Baltimore, MD, USA) discussed the ALS-like phenotype in transgenic mice that express various mutations of the gene for superoxide dismutase 1 (SOD-1). This model gained much attention, although it might only be responsible for a small fraction of the familiar ALS forms. The pathological mechanism is not understood and the working hypothesis now is a certain 'gain of adverse property'.

Many cell-culture systems were presented for neurodegenerative and sensory disorders. However, a problem with cell-line models is that embryonic or immortalized cells such as PC12 are used, which might not represent the situation in adult tissue. Animal models with impressive testing systems are used for peripheral neuropathies[16]. Knockout mice have been examined in almost all systems[17], and many axotomy or mutant-mice models (*pmn*, *wobbler*) have been established for neuromuscular diseases[8,9]. For PD, experimental animal systems such as the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model were presented[11].

Clinical trials

The clinical trials that have been initiated are presented in Table 1. As expected, disorders that are accessible by systemic drug application have been addressed. A detailed analysis of minor side-effects and some clinical improvements in a Phase Ib trial with NGF was presented by Jack Kessler and Stuart Apfel (Bronx, NY, USA). Interestingly, a number of indirect effects and unexpected clinical improvements were observed. Expected side-effects include unwanted transmitter release, hyperinnervation, sprouting of neurons, sympathetic stimulation, induction of antibodies, cachexia, pain and hyperalgesia. Indeed, cachexia and hyperalgesia have been described in CNTF and NGF trials. Reducing the dose seems to overcome such problems, and combinations of drugs might be the method of choice in future. A recently published study showed that the protective effects of neurotrophins might depend on the nature of the insult[18]. Furthermore, neurotrophins were observed to potentiate, rather than inhibit, cell death, which questions the therapeutic use of neurotrophins in certain forms of brain injury.

Drug application

Systemic drug application is only effective for peripheral neuropathies and lower motor neuron diseases. It remains a major challenge to deliver neurotrophic factors to the CNS in a regionally specific, well-tolerated and chronic manner (Box 1). Hefti reported of experience with intracerebro-ventricular pump systems, polymers with slow release and, as did other speakers, some modified, more potent factors. *'Ex vivo'* gene therapy was also addressed (Mark Tuszynski, La Jolla, CA, USA). In rat and primate models of cholinergic neuronal degeneration, genetically modified autologous fibroblasts that produce NGF and are grafted intraparenchymally show a robust and persistent NGF production, ameliorating clinical signs of cognitive deficits without adverse side-effects. Patrick Aebischer (Lausanne, Switzerland) discussed the transplantation of genetically engineered xenogenic cells isolated within a permeability-selective polymer capsule that restricts cell growth and protects from host immune destruction[19]. Localized and safe application of combinations of factors on a continuous basis was achieved in several animal models for neurodegenerative diseases, and significant clinical improvements were observed. A clinical trial has been initiated delivering CNTF to ALS patients using encapsulated genetically engineered cells. Specific targeting of the drugs will be a major problem, as it is for the entire gene therapy approach, and the goals of being able to induce and restrict expression to particular sites are remote.

Concluding remarks

There are still many questions unanswered. Are appropriate combinations of factors more beneficial, are additive or even synergistic effects achieved and can side-effects be thereby reduced? Are the cell and animal models used real predictors for the effects in humans? Which disease is the most appropriate to start clinical trials? Nevertheless, there still remains hope that the next clinical trials with neurotrophic growth factors might be successful and open the field for larger future trials.

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Neurotrophic factors: ready to go?

Nerve growth factor (NGF), the prototype neurotrophic factor, was found several decades ago[1]. It has been shown to promote growth, survival and differentiation of neurons[2–4], and its potential therapeutic use was obvious early on. However, progress in this area has been slow. Within the past few years, a variety of similar factors and their corresponding receptors have been characterized[2,4,5]. These include members of the classical neurotrophin family such as brain-derived growth factor (BDNF), neurotrophin 3 (NT-3), NT-4/5, the ciliary neurotrophic factor (CNTF), members of the fibroblast growth factor (FGF) family, insulin-like growth factor I (IGF-I), transforming growth factor β (TGF-β) and glial-derived growth factor (GDNF), which has been characterized very recently.

Many studies using cell-culture and animal models have shown that neurotrophic growth factors are able to prevent or inhibit neuronal death induced by a variety of insults[6–9]. Can these results be transferred easily to humans, and will they provide sufficient preclinical data for starting clinical trials[10]? A clinical trial using CNTF was unsuccessful because of unexpected side-effects. Therefore, it is important to choose the right factors for the most appropriate patient group with, for example, the correct dosing schemes to demonstrate the clinical feasibility of these molecules in human diseases. The potential use of neurotrophic growth factors as drugs for the treatment of neurological and sensory disorders was the topic of a meeting earlier this year*.

Disorders and neurotrophic factors

Theoretical indications for the beneficial use of growth factors exist for neurodegenerative diseases, brain injuries and peripheral neuropathies resulting from several etiologies. Neurodegenerative diseases are highly devastating disorders, and a characteristic feature of these diseases is the damage and loss of certain neuronal populations[6,7,11]. The ultimate goal would be to protect and rescue these neurons, restore proper function and prevent further progression.

Several neurotrophic factors have been implicated in survival and differentiation of spinal motor neurons[8,9]. Insulin-like growth factor I is effective in various animal models of motor-neuron disorders and no adverse side-effects have been observed in ongoing clinical trials (Jeffry Vaught, West Chester, PA, USA). There is systemic bioactivity allowing subcutaneous application. In the animal models, CNTF or BDNF alone has a moderate effect, but the effects of BDNF and NT-3 are additive, and the effects of BDNF and CNTF are synergistic (Ronald Lindsay, Tarrytown, NY, USA).

One pathological characteristic of Alzheimer's disease (AD) is neuronal loss resulting in deficits in three neuronal systems: cholinergic, hippocampal and cortical neurons[6,7]. Nerve growth factor showed a robust protective effect in the cholinergic system in cell-culture and animal models[6]. This role was reinforced in an elegant study in transgenic mice lacking NGF, where NGF was reintroduced by peripheral expression (Franz Hefti, South San Francisco, CA, USA).

The pathological characteristic of Parkinson's disease (PD) is the loss of dopaminergic neurons in the substantia nigra. Klaus Unsicker (Heidelberg, Germany) discussed the application of FGF-2 and TGF-β and of chromaffin-cell transplants in various models[11]. A particular problem in PD is the difficult nature of the application of growth factors, and, therefore, clinical trials in PD are not yet imminent. Interestingly, in a recent issue of *Nature*, there were four articles addressing the role of GDNF in animal models for PD (Refs 12–15).

Peripheral neuropathies are common and are often debilitating. They involve various neuronal populations, depending upon the particular etiology[16]. Some anticancer drugs (for example, Taxol, vincristine and cisplatin) and diabetes can cause these diseases. By contrast with neurodegenerative diseases, peripheral neuropathies are easier to study because reliable animal models exist for them, they are more accessible allowing systemic drug application, and the character of the disorders is more straightforward. Once again, the goal is to create a more normal phenotype, arrest the disease and stop the progression.

A variety of neurotrophic factors was studied for their effects on sensory deficits resulting from degeneration of sensory receptors or the nerves innervating them. An ideal aim would be to regrow lost receptors. There is a huge demand for treating deficits of the eye and ear, and accordingly, the visual and auditory systems were discussed to a greater extent than the olfactory system. Although remarkable insight into the pathogenesis of these disorders is emerging rapidly and considerable progress has been made, it was obvious that neurotrophic factors as drugs are still far from re-establishing lost function.

The neurotrophic factors that might be effective in particular diseases are shown in Table I (for reviews, see Refs 1–4 and 10). Eventual combination therapies might be difficult to realize since companies are focusing on drugs where they hold the patent rights. However, companies are screening extensively for altered molecules with improved bioavailability, novel factors or receptors. Hefti pointed out that NGF alone might only improve the damage in cholinergic neurons in AD, and hence more than a partial effect of NGF in clinical trials would be surprising. Additional components that target hippocampal and cortical neurons are required to revert the disease fully. One promising factor–receptor combination has been identified by using a screening strategy for novel tyrosine-kinase receptors in the cortex and then characterizing the ligands.

TABLE I. Ongoing or initiated clinical trials

Neurotrophic factor	Disease
Nerve growth factor	Diabetic neuropathy Taxol neuropathy Compressive neuropathy AIDS-related neuropathy Alzheimer's disease (some patients in Sweden)
Brain-derived growth factor	Amyotrophic lateral sclerosis
Neurotrophin 3	Large fiber neuropathy
Insulin-like growth factor	Amyotrophic lateral sclerosis Vincristine neuropathy Taxol neuropathy
Ciliary neurotrophic factor	Amyotrophic lateral sclerosis

Growth Factors as Drugs for Neurological and Sensory Disorders. Held in London, UK: 25–27 April 1995.

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NEUROBIOLOGY

Neuron saving schemes

Ronald M. Lindsay

THE discovery that neuronal loss can be alleviated by neurotrophic factors, such as nerve growth factor, has generated a great deal of interest in the therapeutic potential of these molecules[1–3]. Degeneration of motor neurons in amyotrophic lateral sclerosis (ALS) and of dopamine neurons in Parkinson's disease has been the subject of particular attention in this respect[4,5], and data reported in five papers now open up fresh prospects for research. Four of the papers — by Tomac *et al.*[6], Beck *et al.*[7], Yan *et al.*[8] and Oppenheim *et al.*[9] — appear elsewhere in this issue; the other, by Henderson *et al.*[10], was published late last year in *Science*. Taken together, they indicate that glial-cell-line-derived neurotrophic factor (GDNF) may repay further evaluation for treating neuron loss in both ALS and Parkinson's disease.

The application of molecular techniques to neurobiology has brought about a huge increase in the number of fully characterized neurotrophic factors that promote neuronal survival. In the process, the classical definition of a neurotrophic factor — that of a neuronal-specific, survival-promoting molecule found in limited amounts in the target-fields of responsive neurons — has had to be re-evaluated. This is partly because the prototypical neurotrophic factor, nerve growth factor (NGF), has actions on non-neuronal cells and its synthesis is not restricted to target cells; partly, and more seriously, because it has emerged that certain mitogens and cytokines have NGF-like actions on neurons (see table); and partly because some neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), seem to have paracrine or autocrine functions.

Perhaps surprisingly, given that it was described only two years ago, GDNF was not identified and characterized by state-of-the art molecular techniques, but in the good old-fashioned way — as a conditioned-medium activity that was found to support survival and differentiation of cultured dopamine neurons. Purification, sequencing and cloning revealed it to be a distant member of the transforming growth factor-β superfamily[11]. Faced with the problem of defining a factor on limited data, GDNF was initially taken to be a highly specific neurotrophic factor for dopamine neurons. That it has potent effects on dopamine neurons *in vivo*, as well as in cell culture, finds strong support in the observations of Tomac *et al.*[6] and Beck *et al.*[7]. But its broader actions are clear from the studies of Yan *et al.*[8], Oppenheim *et al.*[9] and Henderson *et al.*[10], all of which show that GDNF has neurotrophic effects on developing motor neurons.

There are various consistencies and discrepancies in the five papers with regard to the neuronal specificity and purported remarkable potency of GDNF. Beck and colleagues[7] confirm that GDNF is a survival factor for cultured dopamine neurons, whereas Henderson *et al.*[10] report the novel finding that GDNF is a survival factor for purified rat embryo spinal motor neurons. Although it is claimed to be more potent than BDNF and neurotrophin 4/5 (NT-4/5), GDNF has in fact a similar efficacy. GDNF is expressed in the developing limb bud, suggesting that it has a target-derived role in motor neuron development. Expression in ventral roots may indicate that GDNF supports motor neurons before target encounter.

A limited survey of the influence of GDNF on neurons of the peripheral nervous system indicates that it does not promote survival of trigeminal sensory neurons or sympathetic neurons. The effects of GDNF do, however, seem to overlap with those of both BDNF and ciliary neurotrophic factor (CNTF) in enhancing survival of nodose sensory neurons. Oppenheim *et al.*[9] report that survival of chick embryo motor neurons in culture is improved by GDNF, with a potency similar to that of CNTF or BDNF. Other data in this paper suggest that GDNF may indeed have effects on sympathetic neurons, as GDNF treatment *in ovo* increases the number of neurons in sympathetic ganglia — implying that these neurons are rescued from programmed cell death. However, GDNF does not reduce programmed cell death in other peripheral ganglia (ciliary, nodose, dorsal root) or in nuclei in the central nervous system, with the exception of motor neurons and interneurons of the isthmo-optic nucleus. Spinal sensory neurons show receptor-mediated retrograde axonal transport of GDNF[8], suggesting that they respond to the factor.

Three reports[8–11] indicate that GDNF affords almost complete rescue of the >80 per cent loss of motor neurons that follows facial nerve axotomy in the neonatal ro-

Activity of neurotrophic factors

	Motor neurons		Dopamine neurons	
Ligand (receptor)	**Survival *in vitro***	**Protect/rescue *in vivo***	**Survival *in vitro***	**Protect/rescue *in vivo***
GDNF (not known)	++++	Neonatal axotomy: survival, no atrophy Adult axotomy: ChAT phenotype	+++	Adult axotomy: survival, TH phenotype MPTP toxicity: protection and recovery of TH, dopamine metabolites, motor behaviours
CNTF (CNTFRα, LIFR, gp130)	++(+)	Neonatal axotomy: survival Efficacy in mice with motor neuron disease: *pmn Wobbler*	–	Adult axotomy: survival
NGF (TrkA)	—	—	—	—
BDNF (TrkB)	++++	Neonatal axotomy: survival Adult axotomy: ChAT phenotype	+++	Adult axotomy: survival, TH phenotype
NT-4/5 (TrkB)	++++	Adult axotomy: ChAT phenotype	++++	Adult axotomy: survival, TH phenotype
FGF (FGFR)	+(+)		Indirect	MPTP toxicity: survival, TH phenotype dopamine
PDGF (PDGFR)	Not known	Not known	++	Not known
IGF-1	Not known	Terminal sprouting	+	Not known

Data mostly taken from refs 1–3. Efficacy *in vitro* runs from – (no effect) to ++++ (almost complete survival). GDNF, glial-cell-line-derived neurotrophic factor; TH, tyrosine hydroxylase; TGF-β, transforming growth factor-β; CNTF, ciliary neurotrophic factor; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-4/5, neurotrophin 4/5; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; IGF-1, insulin-like growth factor-1. ChAT phenotype indicates expression of choline acetyltransferase. TH phenotype indicates expression of tyrosine hydroxylase; MPTP, the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

dent. Uniquely, GDNF seems to prevent both loss of neurons and atrophy of the cell body, though taken alone the clinical relevance of these striking results is debatable. Other studies indicate that the previously reported rescue effects of CNTF (ref. 12) or BDNF (ref. 13) in this rodent model are quite transient[14,15]. Moreover, as a further caution against overinterpretation of animal data, in the *pmn* (progressive motor neuronopathy) mouse mutant, in which CNTF treatment prevents degeneration of motor neurons and thus extends the lifespan of affected animals[16], GDNF rescues motor neurons but has no effect on the premature death of the mice (A. C. Kato and P. Aebischer, personal communication).

Given its initial billing as a highly specific survival and differentiation factor for dopamine neurons, the results of testing GDNF in animal models of Parkinson's disease have been keenly awaited. Tomac *et al.*[6] and Beck *et al.*[7] provide substantial evidence that GDNF does indeed protect or restore function of dopamine neurons compromised by axotomy or the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Although the clinical relevance of their model is questionable, Beck *et al.*[7] clearly show that repeated intranigral injections of GDNF largely attenuate the 40 per cent, or more, loss of dopamine neurons resulting from transection of their axons within the median forebrain bundle. Although this demonstrates the responsiveness of mature dopamine neurons to GDNF, such rescue of axotomized cell bodies does not restore behavioural deficits and so provides only indirect evidence of any clinical usefulness.

However, in a more relevant test of the potential of GDNF to protect or restore dopaminergic function, Tomac *et al.*[6] provide encouraging data from a mouse model of Parkinson's disease produced by the dopaminergic neurotoxin MPTP (which is known to produce parkinsonian symptoms in humans). In one experiment, the authors show that intrastriatal injection of GDNF before administration of MPTP attenuated toxin-induced changes in levels of dopamine and its metabolites, preserved striatal levels of the important enzyme tyrosine hydroxylase, and improved scores in tests of motor function. Furthermore — and this is arguably the most exciting finding — treatment of mice with GDNF days *after* MPTP treatment produced a substantial regeneration of tyrosine-hydroxylase-immunopositive fibres within the striatum, partially restored dopamine levels and improved motor performance.

In all, these effects of GDNF on dopamine neurons are encouraging. But it should be noted that both groups[6,7] resorted to direct intraparenchymal injection of GDNF — this procedure has never been carried out on human subjects, and we don't know about the possible anatomical or behavioural side effects, or how prolonged the restorative effects of GDNF might be.

As the aetiologies of Parkinson's disease and sporadic ALS are unknown, devising ways to arrest these diseases rely largely on broad concepts of how best to interfere with neuronal atrophy and loss. Oxidative stress, excessive Ca^{2+} and glutamate toxicity are high on the list of suspects, but the basic biochemical nature of these processes has made it difficult to prove their guilt or to abolish their deleterious actions on neurons. The striking effects of neurotrophic factors in cell culture and the long list of accomplishments of NGF (and now a variety of other factors) in animal studies makes these molecules attractive candidates as therapeutic agents. But animal models can be poor predictors of adverse reactions in patients. This is highlighted by the confounding side effects of hyperalgesia and weight loss found in clinical trials of NGF (ref. 17) and CNTF (J. M. Cedarbaum, unpublished results), respectively, despite the strong rationale behind these trials and these factors' efficacy in animal studies. Such side effects may, however, be overcome by using combinations of growth factors that have synergetic effects at dose levels that show no side effects[18]. Clinical studies with CNTF, BDNF and insulin-like growth factor-1 (IGF-1) in ALS patients are in progress.

The new reports on GDNF biology highlight how quickly the discovery of ligands is followed by assessment of their therapeutic potential. But further animal studies, and more detail on the distribution of GDNF-binding sites and its general pharmacology, pharmacokinetics, stability and toxicology, are necessary before we can see just how promising that potential is. □

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DAEDALUS

Recycled teeth

LAST week Daedalus was musing on the atomic bomb, in which a h ll w shell of high expl sive is d tonated to c mpress a central charge of plutonium. He wanted t use the technique on samples or assemblies of animal teeth. Under the sudden massive pressure they should flow plastically, and pressure-weld together into an artificial ivory. He is now applying the idea to human teeth.

Restorative dentistry is bedevilled by the lack of any good substitute for tooth material. Gold is expensive, amalgam slowly erodes, porcelain is hard to shape, and none of them bonds firmly to the tooth beneath. But implosive forming, says Daedalus, would permit human tooth substance itself to be formed into crowns and inserts. The dentist would take the usual wax or elastomer impression, but instead of casting a crown from it, its shape would be scanned into a computer. A program adapted from those of bomb physics would then calculate what pattern of explosive charges would deform what initial sample of dentine into that exact shape. The program could even do the calculation for a composite sample, such as a chunk of dentine in contact with a sliver of tooth enamel. The explosion would pressure-weld the two into a single insert of the desired shape, faced with a hard enamel biting surface.

The ideal material for such an insert would, of course, be tooth substance from the patient himself. This would be immunologically compatible with its new site. It should be welcomed back into the body, and should bind firmly into place by slow protein infiltration. But where to get a sample of the patient's tooth substance, without pulling out another of his teeth? Daedalus recalls the 'milk teeth' we all shed as infants, from the age of about six. These teeth should not be discarded or sold to the tooth fairy, but carefully preserved in liquid nitrogen. They would then be available as raw material for subsequent dental repairs to their original owner.

We grow and shed only 20 milk teeth; they are quite small, and have no root (it is resorbed in the shedding process). This modest reserve of material should still provide a lifetime's supply of crowns and inserts, and could even give a whole replacement tooth as well. To fit in the jaw, such a tooth would need a properly shaped root. It would have to be pressure-welded from several milk teeth, and th n forced into its place in the mouth. It should soon bind fast t the compatible tissu all around it, and would then be as good as new. Indeed, it would be even better — having no nerve, it could never ache. David Jones

Neurotrophic factors in diabetic neuropathy

We support the conclusion of Brewster and colleagues[1] that a deficient neurotrophic factor might be implicated in the pathogenesis of diabetic neuropathy, although there is evidence against nerve growth factor (NGF) being the specific factor involved.

The symmetrical sensory loss that affects the longest axons, and which is characteristic of human diabetic neuropathy, also occurs in leprosy[2,3]. In both diseases, sensory loss, particularly involving nerve fibres that mediate pain, leads to trophic ulcers on the feet, causing considerable disability. Some patients with leprosy and diabetes can develop Charcot's joints[4,5] where there is swelling, and excessive painless movement of the tarsal joints with extensive bony destruction. Another characteristic radiological feature that is common to both diseases is a tapering of the heads of the metatarsals and phalanges[6,7]. However, there are important differences between the two diseases in that postural hypotension does not occur in leprosy[2,8], suggesting that vasomotor control, and hence adrenergic function, is not impaired.

Anand *et al.*[9] have described a patient with postural hypotension, and virtual absence of NGF, yet no evidence of trophic ulcers or Charcot's joints. Morphologically, there was loss of small diameter, myelinated, probably Aδ fibres, suggesting that this group is associated with low levels of NGF, a view supported by the fact that when antibodies to NGF are injected into newborn rats there is a specific loss of Aδ fibres[10], but again without any of the complications seen in leprous or diabetic neuropathy.

An autoimmune animal model of nonlepromatous leprosy has been developed in the rabbit using human peripheral nerve as antigen. The electrophysiological findings[11] are shown in the Table. Despite the decreased conduction velocities in Aα fibres, and the diminished amplitude of the C_2 component of unmyelinated fibres, the Aδ component was completely normal. Three rabbits with C_2 abnormalities were found to be analgesic before the electrophysiological studies. The highly selective nature of the abnormalities indicates that an autoimmune response to a neurotrophic factor might have occurred, but because there was no change in either the conduction velocity or amplitude of response of Aδ fibres, this factor is unlikely to be NGF.

TABLE. Altered conduction velocities in an autoimmune animal model of nonlepromatous leprosy

Nerve fibre	Injected with peripheral nerve + adjuvant (8)	Control (5)
Aα	< 42 ms^{-1} (4 rabbits)	42.0 ms^{-1}
Aβ	31.0 ms^{-1}	31.0 ms^{-1}
Aδ	9.9 ms^{-1}	9.9 ms^{-1}
C_1	1.5 ms^{-1}	1.5 ms^{-1}
C_2	1.2 ms^{-1}	1.2 ms^{-1}
	C_2/C_1 <1 (5 rabbits)	C_2/C_1 = 2.85/1

Dutch Bantam rabbits were injected with suspensions of human peripheral nerve plus Freund's Complete Adjuvant in one hind-limb foot pad. The sural nerve of the opposite limb was exposed under biceps femoris, and sectioned at its junction with the sciatic nerve, desheathed and placed on a pair of platinum electrodes. The nerve was crushed between recording electrodes to give monophasic action potentials. Stimulation was administrated in the popliteal fossa. The C-fibre potential invariably showed two peaks, referred to as C_1 and C_2, corresponding to conduction velocities of 1.5 ms^{-1} and 1.2 ms^{-1}, respectively. Note, only the C_2-fibre action potential has been affected. Amplitude of C-fibre action potentials expressed as C_2/C_1 ratio.

The symmetrical sensory loss in leprosy is remarkably selective, involving only superficial sensory modalities[2,3]. The neural-crest cells, that develop into dorsal root ganglion cells, divide early into a ventrolateral group that supplies the skin, and a mediodorsal group, but only the latter is responsive to NGF (Ref. 12). Therefore, it is possible that lack of an unknown growth factor could be implicated in the production of Charcot's joints and trophic ulcers in leprosy, and also in diabetic neuropathy. This growth factor, judging from the results of our animal studies, might be immunogenic, and hence capable of isolation. Use of antibodies might enable the identification of a structural component of peripheral nerve producing the electrophysiological abnormalities.

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We are grateful to J.E. Pascoe for performing the electrophysiological studies.

In a recent article in *TINS*, Brewster and colleagues[1] provided an excellent review of studies using nerve growth factor (NGF) and related neurotrophic factors for treatment of diabetic-induced and other peripheral neuropathies. However, their comments on a study that demonstrated that NGF could prevent the cytotoxic effects of the antitumor drug taxol on dorsal root ganglion (DRG) neurons in explant cultures failed to include the correct citation of this work[2]. Instead, the authors incorrectly attributed this study of taxol-treated DRG neurons to an earlier paper on taxol-treated fibroblasts in culture that did not involve NGF (Ref. 3). The clinical relevance of our studies of the selective rescue of taxol-treated DRG neurons by NGF *in vitro*[2,4] has been well established by recent studies that demonstrate that exogenous NGF can prevent taxol-induced sensory neuropathic changes in adult mice *in vivo*[5], as well as diabetic sensory neuropathy in rats[6]. These results have led to plans for the use of NGF to protect sensory neurons in patients with diabetic neuropathy, and proposals for NGF use during taxol treatment of patients with cancer (J.A. Kessler and S.C. Apfel, pers. commun.).

In another section of their review, Brewster and colleagues note that injections of NGF into adult animals have been shown to elicit hyperalgesic effects[7] that 'could cause problems in the event of therapeutic use of NGF...for neurological disorders'. However, the authors did not comment on a recent study carried out on nociceptive types of DRG

neurons in explant cultures. These studies demonstrated that rapid excitatory effects of NGF on the action potential (which provide an *in vitro* model of NGF-induced hyperalgesia *in vivo*) can be prevented by co-treatment with antagonists of opioid receptors, for example, naloxone or norbinaltorphimine[8]. The specificity of the κ-opioid-receptor antagonist norbinaltorphimine (but not selective, μ- or δ-opioid-receptor antagonists) in preventing NGF-induced excitatory effects appears to be mediated by NGF-stimulated release of endogenous dynorphin from DRG neurons, which acts locally to activate excitatory κ-opioid receptors on the surface of these neurons[9], thereby prolonging the duration of the Ca^{2+}-dependent component of the action potential[10,11]. The clinical relevance of these *in vitro* studies, that suggest that the hyperalgesic side-effects of NGF might be prevented by co-treatment with selective antagonists of excitatory κ-opioid-receptor functions, has been established by subsequent studies *in vivo* that show that naloxone as well as binaltorphimine can, indeed, prevent NGF-induced hyperalgesia in adult rodents[12].

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Reply

We do not believe deficient NGF is the sole cause of diabetic neuropathy, and our review[1] did not suggest such a standpoint. Other neurotrophic factors, including brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin 4/5 could also be implicated in such a multifaceted disease. A recent elegant study by McMahon *et al.*[2] shows that 39% of adult sensory neurones (L_3 in origin), innervating cutaneous tissue, do not express any known Trk receptor, these neurones were all 25 μm or less in cell diameter. This result clearly implies the presence of an unknown growth factor(s) required for maintenance of C-fibre type neurones in adult skin. Another interesting finding from this study was the distribution of TrkB receptors: 16% of L_3 neurones expressed *TrkB* and all of these also expressed either *TrkA* or *TrkC*. We have recently measured deficient levels of BDNF mRNA in foot-skin of eight-week diabetic rats (unpublished data). Such co-operativity with regard to *Trk* expression indicates that changes in BDNF expression could be detrimental to a wide range of sensory neurone types.

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Nervous Excitement over Neurotrophic Factors

Cephalon's success with IGF-1 has put neurotrophic factors back in the biopharmaceutical spotlight

Gary Walsh

The positive phase III clinical results in mid-June from Cephalon's (West Chester, PA) Myotrophin (recombinant human IGF-1) in amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) signaled resurgent interest not only in neurotrophic factors but also in early-stage biotechnology companies in general. In a trial involving 266 patients, Cephalon showed convincingly that those receiving Myotrophin experienced less disease severity, slower progression of disease, and better functional ability compared to those given placebo. Cephalon is developing Myotrophin in North America and Europe for ALS in collaboration with Chiron (Emeryville, CA) and in Japan for ALS and other neurological disorders with Kyowa Hakko Kogyo (Tokyo). The positive news was particularly welcome, since ciliary neurotrophic factor (CNTF) from both Regeneron (Tarrytown, NY) and the Syntex-Synergen (Palo Alto, CA; Boulder, CO) neuroscience joint venture had failed in phase III trials in 1994 and 1995, respectively, after promising preclinical and early clinical data.

Neurodegenerative diseases are characterized by the death of specific neuronal populations. There are no effective therapies for any neurodegenerative diseases: Consequently, many companies are pursuing neurotrophic factors as potential therapies for "unmet medical needs" (Table 1).

Neurotrophic Biopharmaceuticals: The Potential

Many neuronal cell types are responsive, *in vitro* at least, to one or more neurotrophic factors (Table 2). Until Cephalon, however, disappointing clinical trial results had forcefully underlined the fact that preclinical data does not necessarily portend physiologically significant responses in humans.

Neurotrophic factors are regulatory molecules that play a central role in the development, survival, and maintenance of neurons.[1,2,3] While the first—nerve growth factor (NGF)—was identified almost a half-century ago—it is only in the last five years that many others have been characterized. Specific neurotrophic factors normally sustain or otherwise influence various neuronal populations, including those affected by neurodegenerative diseases.

Neurotrophins or Neurotrophic Factors?

Several neurotrophic factors belong to a single gene family, the neurotrophins.[1] These include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (NT-6). All are small, basic proteins, sharing approximately 50% amino acid homology, existing mainly as homodimers, and activating signal pathways by binding to a member of the Trk family of tyrosine kinase receptors (Table 2).[3] Each neurotrophic factor influences development and maintenance of a specific set of neurons, with some cells being sensitive to more than one such factor or factors. Additional neurotrophic factors include CNTF and glial cell line-derived neurotrophic factor, GDNF.

Insulinlike growth factors I and II (IGF-I, IGF-II), fibroblast growth factors (FGF), platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF-β1), granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO), and leukemia inhibiting factor (LIF)—all regulatory molecules previously known for their effects on nonneuronal tissues— also act on neurons.

The classical understanding of a neurotrophic factor is as a molecule, usually synthesized and released in limited quantities from a neuron's target tissue, that in effect nourishes and guides sensitive neurons. However, that understanding has had to be

Neurotrophic factors are regulatory molecules that play a central role in the development, survival, and maintenance of neurons.

Company	Products in Development (Indication)
Alkermes Inc. (Cambridge, MA)	NGF-transferrin (Alzheimer's), NGF (Alzheimer's, Parkinson's)
Amgen (Thousand Oaks, CA)	BDNF (ALS, motor neuron disease), GDNF (Parkinson's, ALS)
Cephalon (West Chester, PA)	IGF-1 (ALS), NGF/IGF-1 (Peripheral neuropathy)
Cyto Therapeutics Inc. (Providence, RI)	CNTF (ALS)
Genentech (S. San Francisco, CA)	NGF/IGF-1 (Peripheral neuropathy), NT-4/5 (ALS, Parkinson's, Alzheimer's), GDNF (Parkinson's, ALS)
Regeneron Pharmaceuticals	CNTF (ALS), BDNF (ALS)
Syntex Inc. (Palo Alto, CA) Synergen (Boulder, CO) joint venture	CNTF (ALS), GDNF (Parkinson's)

TABLE 1. Some of the main players in the neurotrophic factors game.

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TABLE 2. Molecules with neurotrophic activity in vivo and/or with neurons in culture, and the Trk receptors of neurotrophin tyrosine kinase with which they interact.

Neurotrophic Factor	Biology	Cells Influenced	Potential In:	Receptor
Nerve growth factor (NGF)	2 x 118 aa	Sympathetic neurons; cholinergic basal forebrain neurons	Alzheimer's, peripheral neuropathies	Trk A
Brain-derived neurotrophic factor (BDNF)	119 aa; pI 9.9; localized in CNS	Retinal ganglia, dopaminergic neurons, cholinergic neurons, embryonic spinal motor neurons, cortical neurons	ALS, Parkinsonism, Huntington's	Trk B
Neurotrophin-3 (NT-3)	119 aa; pI 9.5; expressed in embryogenesis	Various, including dorsal root and nodose ganglia neurons, neuropathies	peripheral	Trk A, Trk B, Trk C
Neurotrophin-4/5 (NT-4/5), mammalian form		Various motor neurons, various CNS neurons	ALS, Alzheimer's, Parkinson's, Huntington's	Trk B
Neurotrophin-6 (NT-6) (from *Xiphophorus* fish)	143 aa, pI 10.8	To be fully characterized. Spectrum of sensitive neurons appear similar to those of NGF.		?
Ciliary neurotrophic factor (CNTF)	200 aa; acidic cytoplasmic protein; released from damaged cells	Ciliary neurons; spinal motor neurons	ALS, peripheral neuropathies	Trimer, consisting of CNTFRα, LIFR ß, and GP13
Glial cell line-derived neurotrophic factor (GDNF)	Homodimeric glycosylated polypeptide; 34-45 kDa	Dopaminergic, developing motor neurons, spinal sensorimotor neurons	Parkinsonism, ALS	?

reevaluated both because recent studies of many of the molecules listed above have shown both that neurotrophic factor synthesis is not restricted to target cells and that some factors affect nonneuronal cells. NGF, for example, is synthesized by mast cells[4] and may influence cells of the immune system.

Furthermore, some neurotrophins appear to display paracrine or autocrine effects.[5] Sensory neurons of the dorsal root ganglion (DRG), for example, lose their dependence on exogenous growth factor (S54) upon maturation.[6] The mature cells express both BDNF and its receptor, suggesting an autocrine role for the neurotrophin. Several neuronal populations are believed to express specific neurotrophins and their receptors.[6]

A better understanding of the physiological significance of neurotrophic factors regarding neuronal function may also be obtained by studying "knockouts"—animals in which the genes coding for specific neurotrophic factors (or their receptors) have been deleted.[7]

Neurotrophic Biopharmaceuticals: The Reality

One of the first clinical targets for neurotrophic factors was amyotrophic lateral sclerosis (ALS, also

How Nerve Cells Grow

The nervous system consists of three cell types: neuronal, (neuro)glial, and endothelial cells. The neurons constitute the communicative elements, while the glial cells serve various supportive roles. The central nervous system (CNS), which integrates all neuronal activities, consists of the brain and spinal cord, while the additional vertebral neuronal elements constitute the peripheral nervous system (PNS).

The cytoskeleton of neuronal cells provides the axon—which may be several meters long in large mammals—with mechanical support and plays a critical role in the transport of materials from the cell body toward the synapse (anterograde transport) and away from it (retrograde transport). The axons of most mature neurons are covered with a myelin sheath, formed by specific glial cells (oligodendrocytes in the CNS and Schwann cells in the PNS). Sensory neurons are those leading from a stimulus-detecting receptor cell, while motor neurons are those normally carrying a nerve impulse to an effector cell, often a muscle cell.

Axonal growth toward its target tissue appears to be mediated by specific guidance molecules leading toward the target tissue for which specific receptors exist on the surface of the axonal growth cone. Such guidance molecules include surface glycoproteins (cell adhesion molecules), which provide direct cell-cell and other guidance, and soluble (diffusable) chemoattractants.

If the developing axon fails to innervate the target tissue, the neuronal cell dies; furthermore, many (excess) neurons also die upon innervation. Electrical activity is important in determining which neurons survive but so is competition between neurons for soluble target-derived factors such as neurotrophic factors. These are required to develop, guide, and maintain the innervating neuron.

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known as Lou Gehrig's disease or motor neuron disease), a neurodegenerative condition characterized by the degeneration of spinal and brainstem motor neurons. ALS results in muscle wasting, and eventually death, and affects up to 70,000 people worldwide, representing an estimated $450 million market in the U.S. alone.

Several neurotrophic factors are known to positively influence motor neurons in vivo and in vitro. Some of the earliest work in ALS, pioneered by Regeneron, Synergen and Syntex, was with CNTF. CNTF[8] influences a broad range of neuronal cells, including spinal motor neurons. Its distribution and developmental expression, and the fact that it is a cytoplasmic protein, suggest that it is not a target-derived neurotrophic factor.[9] It probably exerts its neurotrophic and differentiative effects after being released from damaged cells.

The CNTF clinical results were initially encouraging but ultimately disappointing. Regeneron's phase III trial foundered early in 1994, and Syntex and Synergen discontinued their ALS clinical trial in February 1995 because of lack of efficacy.

Although Cephalon has demonstrated the effectiveness of IGF-1 in ALS, little is known still about its precise mechanisms of action. In preclinical studies, IGF-1 was shown to support the survival of motor neurons, to accelerate the regeneration of damaged neurons, to promote sprouting and function of peripheral nerves, and to induce enlargement of muscle cells, all under neurodegenerative conditions. In neuronal sprouting, neurons generate additional branches that allow them to establish functional contacts with muscle fibers whose original nerve contacts have been lost. Cephalon suggests that these manifold actions of IGF-1 may explain the effects of Myotrophin in its trial. Cephalon has recently concluded a second phase III clinical study comparing Myotrophin to placebo in 183 patients with ALS in Europe.

The neurotrophic quest to conquer ALS does not end with IGF-1, however. Based on a preliminary analysis of a 283-patient phase I/II trial, Amgen and Regeneron began their phase III trial of BDNF in the third quarter of 1995. Trials of GDNF are also planned. GDNF is the most potent known survival factor for motor neurons in vitro,[10] and influences both developing and adult motor neurons in vivo.[5]

Likely future strategies will also entail using a combination of neurotrophins on the basis of recent work by Mitsumoto and coworkers,[11] who found that simultaneous subcutaneous administration of CNTF and BDNF arrested progression of motor neuron dysfunction in *Wobbler* mice (animal model of motor neuron disease). Administration of either factor on its own only slowed progression of the disease.[11, 12]

The clinical relevance of administering neurotrophic factors in combination rather than singly in the treatment of ALS, or indeed other neurodegenerative diseases could also be appraised by using genetically engineered molecules exhibiting multiple neurotrophic activities. Pan-Neurotrophin-1 (developed by Regeneron in collaboration with the Karolinska Institute, Sweden)[13] is an engineered chimeric neurotrophin containing the active domains of NGF, BDNF, and NT-3, which can bind the neurotrophin receptors P75, Trk A, Trk B and Trk C.

Other Targets for Intervention

Peripheral neuropathy represents another target for neurotrophic intervention. This disease is characterized by the degeneration of sensory and motor neurons. It is associated mainly with diabetes but is also seen in some cancer patients receiving certain forms of chemotherapy. Peripheral neuropathy often warrants amputation of limbs affected by neuronal loss.

Peripheral sensory and sympathetic neurons, depleted in peripheral neuropathy respond to NGF which, along with IGF-1, can prevent drug-induced peripheral neuropathies in animals. Cephalon has initiated a phase II clinical program to evaluate Myotrophin's use in treating a variety of peripheral neuropathies, including chemotherapy-induced peripheral neuropathy, post-polio syndrome, and diabetic neuropathy. Genentech is currently in phase II clinical trials with NGF in peripheral neuropathy.[14, 15]

Interestingly, certain neurons involved in mediating pain and thermal receptive functions are also responsive to NGF. Manipulation of NGF signal transduction thus could also have potential in the control of pain.

Delivery Is a Prerequisite to Progress

Diseases such as Alzheimer's and Parkinson's are characterized by the death of specific brain cells. Unlike diseases affecting peripheral neurons, clinical intervention in these cases is rendered complicated by the presence of the blood-brain barrier (BBB), which prevents neurotrophic factor administration by intravenous means. While many animal studies involve direct injection of neurotrophic factors into the brain, this approach is unlikely to be adopted in human therapy. Development of safe and effective delivery systems is a prerequisite to clinically relevant progress in this area. The use of infusion pumps is possible, but hardly ideal. One delivery strategy currently being developed by Cyto Therapeutics (Providence, RI) involves intraventricular transplantation of polymer-encapsulated cells producing recombinant neurotrophic factors.[16]

An alternative delivery strategy pioneered at Alkermes (Cambridge, MA) entails conjugating neurotrophic factors to antibodies recognizing the transferrin receptor, which functions to transport iron across the BBB.[17] Research shows that NGF-anti-transferrin antibody conjugates can cross the BBB, and studies in rats have shown that, upon entry into the brain, the conjugate can promote the survival of NGF-sensitive neurons, including the cholinergic striatal interneurons, which degenerate in Huntington's disease.[18]

Improvements in Memory and Motor Functions

Alzheimer's disease is characterized by loss of the cholinergic neurons of the basal forebrain that enervate the hippocampus (the brain's memory center). These cells are sensitive to NGF, and NGF can rescue such cells in rodents after damage induced by cutting their axons. NGF, therefore, represents a poten-

One of the first clinical targets for neurotrophic factors was amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig's disease or motor neuron disease), a neurodegenerative condition characterized by the degeneration of the spinal and brainstem motor neurons.

Parkinson's disease is one condition for which neurotrophic factors may prove useful.

Neurotrophic Factors and Their Receptors

Most if not all the biological activities of the neurotrophins are mediated by their binding to high affinity receptors on the surface of susceptible cells. These receptors belong to a family of receptor tyrosine kinases, the Trks, which are similar to tyrosine kinase receptors of other growth factors but are expressed almost exclusively in the nervous system. Three distinct receptors, Trk A, Trk B, and Trk C have been characterized (see Table 2). Binding the appropriate neurotrophin induces Trk dimerization, resulting in its autophosphorylation (activation), thus initiating signal transduction.

In addition to the high-affinity receptors, many neuronal and nonneuronal cells express low-affinity NGF receptor (LNGFR or P75), which apparently serves as a low affinity receptor for all the neurotrophins. The precise role of P75 is uncertain but its presence on nonneuronal (including glial) cells suggests that it may bind and "present" neurotrophins to developing axons bearing high-affinity receptors, thereby directing axonal growth toward its target during development. P75 belongs to a superfamily of TNF receptors, some of which are known to regulate apoptosis in some cells: P75 possibly plays a similar role in neurons.

The trimeric ciliary neurotrophic factor receptor, though unrelated to the neurotrophin receptor family displays significant homology to the interleukin-6 receptor. The GDNF receptor remains to be characterized.

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tial therapeutic agent in the treatment of Alzheimer's disease, and results from clinical trials planned by the Synergen/Syntex partnership are keenly awaited. NGF infusion into one Alzheimer's patient was reported to result in memory improvement.

Degeneration of forebrain cholinergic neurons is also associated with age-dependent decreases in learning and memory abilities. Infusion of NGF, NT-3, and NT-4/5 may reverse spatial memory impairment in aged rats, which is associated with a

reduction in cholinergic neuronal atrophy.[19] A U.S. team working with Cyto Therapeutics has also shown recently that NGF can rescue damaged cholinergic basal forebrain neurons in aged monkeys.[16]

These results, taken together, indicate that NGF, and possibly additional neurotrophic factors, may be of clinical interest in retarding progressive memory and learning impairments associated with aging. Interestingly, researchers at the University of California, Irvine, have recently shown that physical exercise in rodents induces increased BDNF expression.[20] Since BDNF also supports forebrain cholinergic neurons, this may explain in part why exercise is considered a predictor of high mental function in aging.

Parkinson's disease, characterized by the degeneration of dopaminergic neurons of the substantia nigra also represents a condition for which neurotrophic factors may prove useful. NT-4/5 and BDNF are known to promote survival of these cells *in vitro*, but GDNF may prove most useful.

GDNF, first characterized in 1993,[21] may yet prove to be the neurotrophic factor with the most significant therapeutic potential. It is a member of the TGF-β superfamily and appears to influence a wide range of neuronal populations.[5, 10] In animal models of Parkinson's disease, direct repeated injection of GDNF to sites adjacent to the substantia nigra not only prevents dopaminergic loss following physical or chemical damage but even promotes significant restoration of the nigrostriatal dopamine system. Thus, GDNF might play a potential therapeutic role in halting or even reversing Parkinsonism.[5]

Neurotrophic factors, therefore, have potential application in the treatment of some of the most distressing diseases affecting humankind. Only time, and clinical trials, however, will show whether that potential can be realized.

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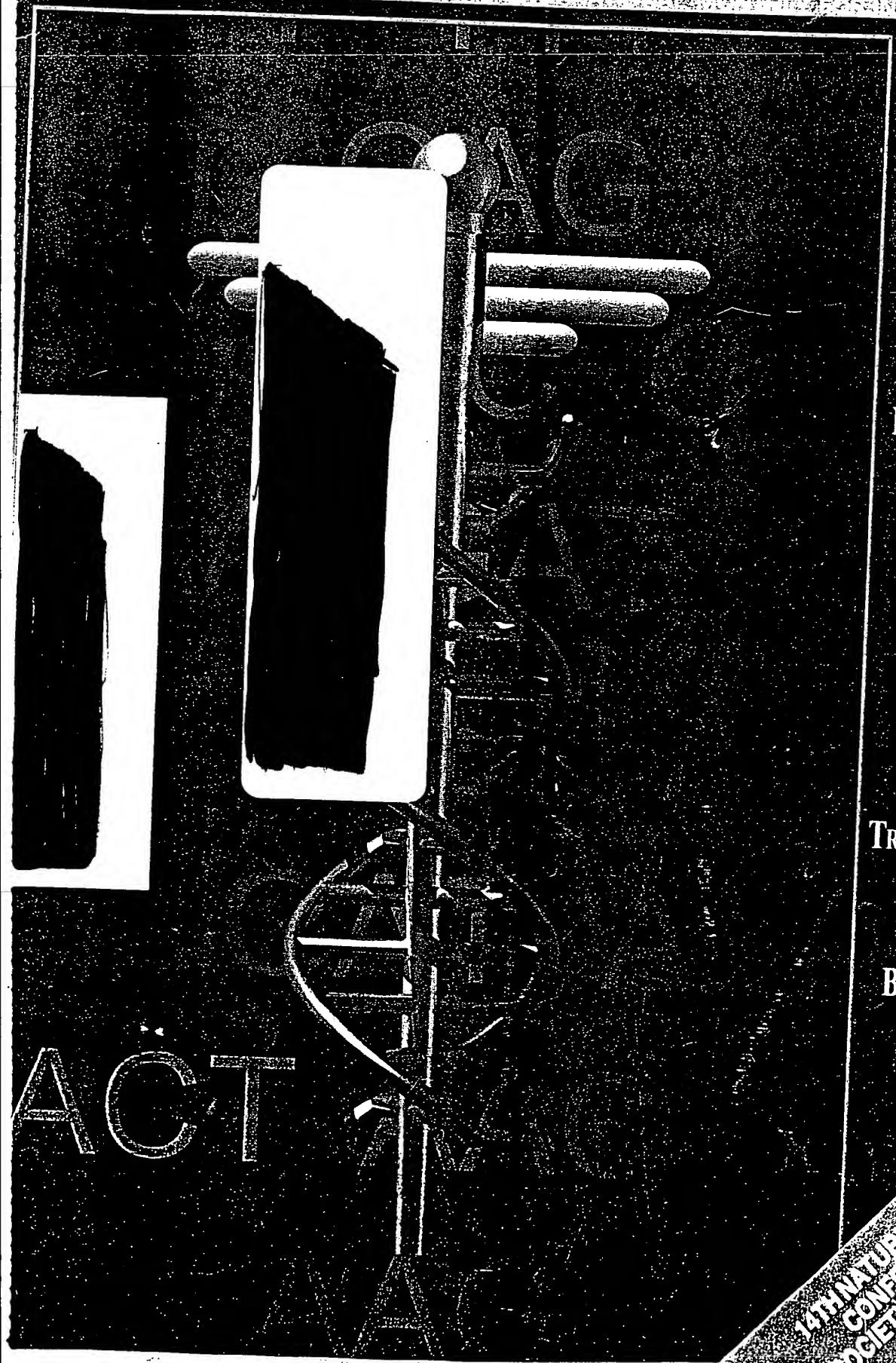
NOVEMBER 1995



THE NEXT SEQUENCE OF GENOMIC PARTNERSHIPS

NERVOUS EXCITEMENT OVER NEUROTROPHIC FACTORS

MAMMALIAN CELL TECHNOLOGY: SECRETION ON DEMAND

TRANSDERMAL PROTEIN DELIVERY

BIOTECHNOLOGY OF BREADMAKING

14TH NATURE BIOTECHNOLOGY
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SOCIETY FOR NEUROSCIENCE
SAN DIEGO
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Neurotrophic Factors Enter the Clinic

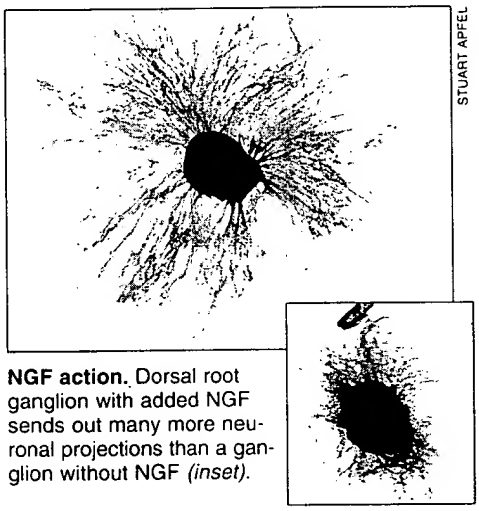
The biotech industry launches a new class of nerve-nurturing drugs with high hopes of toppling stubborn neurological diseases such as Lou Gehrig's disease

Neurons are among our most precious cells. They play vital roles in our lives, governing everything from the recoil of a finger from a hot stove to the understanding of Dante. Unlike the vast majority of other cells, however, most neurons must perform these all-important tasks for an entire lifetime, as they aren't replaced if destroyed by injury or disease. Given that neurons are so precious, it's not surprising that one of the hottest areas in neuroscience today is the study of neurotrophic factors: naturally occurring proteins that keep neurons alive and healthy during embryonic development and later in normal adult life.

This interest in neurotrophic factors is not merely academic. In fact, it has already generated a "wave of excitement" in the biotech world, says Jeff Vaught, senior vice president for research at Cephalon, a biotech company in West Chester, Pennsylvania. A half-dozen or more companies have clinical trials planned or under way for testing neurotrophic factors against debilitating neurodegenerative diseases, including Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease.

"Most of the diseases for which these neurotrophic factors are being developed are diseases for which current therapies are virtually nonexistent," says biotech stock analyst Margaret McGeorge of Hancock Institutional Equity Services in San Francisco. That lack of treatments, she says, is a driving force behind the current enthusiasm. But McGeorge also points out that "it is much too early to say" which factors will pan out as useful drugs. One highly touted neurotrophic factor has already stumbled in clinical trials—possibly a result of being rushed into large-scale trials. That experience hints that bringing these nerve-nurturing drugs to market could pose bigger challenges than companies originally thought. Still, that setback has only slightly dimmed the excitement that surrounds neurotrophic factors in the research and biotech communities.

That excitement has been building to its current crescendo for a long time, beginning more than 30 years ago with the Nobel prize–winning discovery of the first neurotrophic factor—nerve growth factor (NGF)—by Rita Levi-Montalcini, Stanley Cohen, and Viktor Hamburger at Washington University in St. Louis. They showed that NGF ensures the survival of certain peripheral neurons as they grow toward and connect with target tissues during the development of the nervous system. Later work showed that NGF is needed for the survival of some brain neurons as well.



STUART APFEL

NGF action. Dorsal root ganglion with added NGF sends out many more neuronal projections than a ganglion without NGF *(inset)*.

But many types of nerve cells didn't respond to NGF, raising the possibility that they are supported by other factors. The search for those factors has so far uncovered roughly a dozen proteins that help neurons survive throughout the body and brain. Some, like NGF, work only on some types of nerve cells. Others, such as insulin-like growth factor (IGF) and fibroblast growth factor (FGF), were originally identified as growth factors for other tissues, such as muscle, or the immune system, and were only later found to nurture neurons as well. As the field has grown, the definition of a neurotrophic factor "has been expanded a lot," says Washington University neuroscientist Eugene Johnson, "and it's clear that there are a lot more players."

ALS: A cautionary tale

As this cast of characters expanded, researchers quickly realized that some of the newly discovered growth factors might serve as therapeutic agents for neurological diseases. The first disease to be targeted by neurotrophic factors in a major clinical trial was ALS, a deadly condition in which the motor neurons—neurons that control the skeletal muscles—progressively degenerate, eventually depriving patients of all movements, even the ability to breathe.

ALS was a good starting point for several reasons. For one thing, there's no effective treatment for the disorder. In addition, motor neurons, unlike neurons of the brain, are accessible to protein drugs introduced into the bloodstream. Says Cephalon's Vaught, diseases like ALS should be "very amenable to growth-factor application because the projections from the affected neurons lie outside the blood-brain barrier," the physiological wall that keeps large molecules like proteins from entering the brain.

A number of growth factors had already been shown to support the growth of motor neurons in laboratory cultures, and three of those factors became early candidates for treating ALS: brain-derived neurotrophic factor (BDNF) and insulin-like growth factor I (IGF-I), both made by muscles controlled by motor neurons, and ciliary neurotrophic factor (CNTF), made by the Schwann cells that form an insulating sheath around the neurons.

In addition to the cell-culture work, all three factors also turned out to aid the healing of injured motor neurons in rats. Moreover, the factors improved the condition of mice with hereditary motor-neuron diseases similar to ALS. That was enough for two companies, Regeneron Pharmaceuticals of Tarrytown, New York, and Synergen of Boulder, Colorado, to begin clinical trials of CNTF and for a third, Cephalon, to begin trials with IGF-I. Regeneron is also developing BDNF, in collaboration with Amgen of Thousand Oaks, California, although they have not yet begun large-scale trials.

But the high hopes with which these trials began quickly turned into what many observers view as a cautionary lesson for the field. The first publicly reported results with CNTF looked promising enough: Last September, at the World Congress of Neurology in Vancouver, British Columbia, Regeneron announced that 12 ALS patients who had been receiving CNTF injections in a small, preliminary trial showed less of a decline in muscle strength than did 14 patients receiving a placebo. The results were not statistically significant, Jesse Cedarbaum, vice president for clinical affairs at Regeneron, told *Science* in an interview last November, but they were sufficiently encouraging to justify a full-scale trial of CNTF's effectiveness. Last year Regeneron began that trial, which includes 720 patients at 36 clinical sites.

But even as Regeneron trumpeted its ear-

ly results, optimism about CNTF had already begun to erode. Rumors were circulating that patients in a preliminary safety trial of CNTF carried out by Synergen had suffered side effects, including coughing, fever, weight loss, and activation of herpes virus, which can hide in latent form in some neurons.

Similar side effects were also rumored to be plaguing Regeneron's large-scale trial—rumors that were soon to be borne out. In March, Regeneron researchers announced that they had unblinded the data on 550 patients who had completed 6 months of the trial and found that a substantial number of those receiving CNTF had not only had serious side effects, but had actually fared worse on measures of muscle strength than did patients receiving placebos. Patients who didn't experience side effects showed modest improvement as compared to controls, but investors weren't mollified by that scrap of good news, and Regeneron's stock dropped 50%. The company has tried to salvage the trial by applying to the Food and Drug Administration to continue it with lower doses aimed at reducing the side effects.

But the question remains whether anything more than modest effects can be expected of CNTF. Synergen has a large trial in progress, using lower drug doses than Regeneron, based on the findings of its safety trials. The trial has not been unblinded, but insiders say the prospects don't look very exciting. "Although all the numbers haven't been added up [for either large-scale trial], the clinical results in ALS are less than we had hoped for," says Tufts University neurologist Theodore Munsat, who has participated in the Synergen trials. The best that can be expected, says Munsat, is "a modest slowing of the deterioration rate." He questions whether such modest effects would improve the quality of a patient's life enough to justify use of the drug.

But some researchers think the first round of trials is not a fair test of CNTF. Neurologist Michael Sendtner of the Max Planck Institute for Psychiatry in Martinsried, Germany, argues that the way the drug is administered could be key to reducing its side effects and improving its effectiveness. He recently found, for example, that CNTF has a half-life of only 3 minutes in the bloodstream of a rat. That means the injected drug has little time to reach the motor neurons. While in the bloodstream, it encounters tissues such as the liver and lungs and also interacts with blood-borne immune cells. CNTF's interaction with these cells and tissues, which don't usually encounter the factor, may account for its side effects, says Sendtner. He suggests there is still hope for better results if CNTF were delivered directly to the cerebrospinal fluid, which bathes the spinal roots of the motor neurons.

Even if CNTF doesn't pan out, biotech researchers hope IGF-I will have fewer drawbacks as an ALS therapy. That drug has already been used in large-scale drug trials for conditions including diabetes, dwarfism, and osteoporosis without showing serious side effects. "The good news about IGF-I is that it certainly appears so far to be fairly benign," says biotech analyst Timothy Wilson of the New York securities firm Hambrecht & Quist. "But the big question mark is whether or not it will have efficacy in ALS." The Cephalon trial aims to answer that question.

But even if other companies do hit pay dirt with an ALS treatment, close observers of the field think Regeneron's experience holds some lessons for all the companies involved. Some argue, for example, that Regeneron moved precipitously, relying on results from early trials that were too small for adequate analysis of optimal dosing. "Whe companies have tried to move too quickl through these clinical trials, they usually fin disappointment," says R. Brandon Fradd, biotech stock analyst with Montgomery Se curities in San Francisco. But Fradd and oth ers emphasize that, despite the first disap pointing results, the field of neurotrophi factors still shows promise.

Peripheral neuropathy: B tter odds

Some of that promise may be realized in tackling a more straightforward problem. One reason finding ALS therapies is so difficult is that the cause of the motor neuron degeneration remains unknown, so there is no good animal model for the disease. The story is different for peripheral neuropathies: the deterioration of sensory or motor neurons

Solving the Delivery Puzzle

Neurobiologists have high hopes that neurotrophic factors, naturally occurring proteins that help keep nerve cells healthy, may one day provide effective therapies for a wide variety of neurodegenerative diseases (see main text). But before that day comes, they will have to solve a problem: how to administer the factors safely and effectively. It's unlikely that the factors will ever be available as pills that can be washed down with a gulp of water, because they are proteins and thus would be rapidly destroyed in the digestive tract before they could be absorbed. For diseases of the peripheral nerves, the drugs may be injected into the patient's skin, but for brain conditions such as Alzheimer's or Parkinson's disease they may need to be pumped directly into the brain to circumvent the blood-brain barrier, which keeps proteins carried by the bloodstream from reaching the brain.

Growing in. GDNF in capsule attracts growing neurons.

But even though these approaches are being used in the early clinical trials, each has inherent disadvantages. Injecting a trophic factor into the skin sends it into the bloodstream, allowing it to influence many tissues, possibly causing unwanted side effects. And a catheter pumping a trophic factor directly into the brain could be a conduit for dangerous infections. That's why researchers are working to develop more sophisticated delivery systems.

One possibility, under development at CytoTherapeutics Inc. in Providence, Rhode Island, involves enclosing neurotrophic factor–producing cells in tiny, semipermeable capsules that can be implanted anywhere in the body. The capsules—which have been tested in animals and humans—can be implanted in the brain or in the cerebrospinal fluid at the base of the spine. They let nutrients and cellular products pass in and out, but they keep the cells contained and isolated from the recipients' immune defenses. "You can almost think of it as a biological pump," says biotech analyst Margaret McGeorge of Hancock Institutional Equity Services in San Francisco. "You have got a little factory in there, churning out [trophic factor]."

Another strategy is to engineer a person's own skin cells to produce a neurotrophic factor and then tranplant the engineered cells into the brain—a method being tested in rats and monkeys by Fred Gage at the University of California, San Diego. Or the protein factors might be joined with molecules that help them slip across the blood-brain barrier. That approach, being developed by scientists at Alkermes Inc. in Cambridge, Massachusetts, would allow trophic factors injected into the skin to still reach the brain. With these and other approaches in the pipeline, researchers have a rich supply of potential tools to get these nourishing factors to diseased neurons that need them to survive.

–M.B.

caused by diabetes or by treatment with cancer chemotherapeutic agents such as vincristine, cisplatin, and taxol. For those conditions, there are good models. "You can cause peripheral neuropathy with vincristine in a rat and in man," says Wilson, "and if IGF-I prevents it in the rat, there is a very good chance it will do so in man, because it's the same chemical insult."

And this condition has already been met, since IGF-I does prevent drug-induced peripheral neuropathies in animals, as does NGF. Jack Kessler, Stuart Apfel, and their colleagues at Albert Einstein College of Medicine in New York have collaborated with Cephalon and with Genentech of South San Francisco to show the effectiveness of these two factors against drug-induced neuropathies in mice.

As in the rest of this field, clinical trials are following very quickly behind laboratory results; Cephalon is planning to begin clinical trials of IGF-I for peripheral neuropathy within a few months. Genentech has already completed a small multicenter dosing and safety trial of NGF in human patients with peripheral sensory neuropathy, and expects to begin a larger trial by this summer, which would include diabetics with sensory neuropathy. They are tackling diabetes first, says Kessler, who is participating in the studies, because "diabetic neuropathy is a much more prevalent and disabling medical problem" than neuropathy from cancer chemotherapy. As a result of neuropathy, diabetics often experience ulceration of their feet and legs, which can eventually require amputation.

But those two neurotrophic factors aren't the only ones being tried out against neuropathy. Genentech scientist Gao Wei-Qiang recently demonstrated that a factor called neurotrophin-3 (NT-3) protects mice from neuropathy caused by the anti-cancer drug cisplatin, says Franz Hefti, director of Genentech's neuroscience department. The upshot, says Hefti, is that "for peripheral neuropathy a combination of neurotrophins is likely to do the trick."

Parkinson's and Alzheimer's: A fresh approach

Most of the clinical trials now underway have concentrated on testing neurotrophic factors as treatments for conditions such as ALS and peripheral neuropathy in which peripheral nerve cells deteriorate. But that kind of therapy doesn't exhaust the possible uses of the trophic factors. One other important area where these proteins are now beginning to play a role is as experimental therapies for neurodegenerative diseases of the brain.

Alzheimer's is the most prevalent of those diseases, and the leading candidate for Alzheimer's therapy is NGF. An early clue that NGF might be of benefit came from experiments on one of the major groups of neurons that degenerate in Alzheimer's—the cholinergic neurons. These neurons (so called because they release acetylcholine as a neurotransmitter) send their projections into a memory center in a brain structure called the hippocampus. Several research groups have shown that when cholinergic neurons in rat brains are damaged by having their projections cut, they can be saved from dying by infusions of NGF.

Further experiments suggest that saving those neurons may have consequences for mental function. When Fred Gage and coworkers at the University of California, San Diego, infused NGF into the brains of aging rats that showed memory impairment, they found what Gage describes as "very good improvement in [learning] behavior." On a test of memory retention, he adds, the NGF-treated rats "were in the range of the aged nonimpaired group."

Encouraging as these results are, it's far too soon to imagine that NGF will do the same thing for Alzheimer's patients that it did for aging rats. For one thing, the rats are not a perfect animal model for Alzheimer's. Furthermore, it isn't clear whether the decay of the cholinergic neurons is in fact a cause or an effect of human Alzheimer's. Nevertheless, the animal results suggest that keeping the cholinergic neurons alive can improve mental function, says Genentech's Hefti.

Given that possibility, Alzheimer's researchers are ready to see what effects the neurotrophic factor might have in human beings. Lars Olson and his colleagues at the Karolinska Institute in Stockholm have already tried NGF infusion in one Alzheimer's patient; they report that the patient showed improvement on a memory test. Both Genentech and Synergen, the latter in collaboration with Syntex, are planning clinical trials of NGF for Alzheimer's disease as soon as next year. "We don't expect NGF to cure the disease," says Hefti, "but we expect NGF to have a significant behavioral effect."

Researchers also have high hopes for neurotrophic factors in the treatment of Parkinson's disease, another common and intractable neurodegenerative disease, which is characterized by the degeneration of certain dopamine-producing neurons in the brain. Last year, Frank Collins and his colleagues at Synergen purified a protein they call glial cell-line derived neurotrophic factor (GDNF), which supports the survival, in cell culture, of the neurons that die in Parkinson's disease. Using GDNF, "we are hoping that we can reverse the disease," says Synergen chief executive officer Larry Soll. GDNF won't bring a dead neuron back to life, he notes, but it might do something almost as good: Experiments in rats and mice by Synergen researchers suggest GDNF can coax surviving neurons to send out new projections to replace those from lost neurons. If it has similar effects in primates, Soll says Synergen hopes to have GDNF in clinical trials by 1995.

Although the future of any single therapeutic approach with neurotrophic factors remains uncertain, the possibilities are numerous enough to fuel several generations of clinical trials, using increasingly sophisticated delivery systems (see box). And even if the first round comes up short of expectations, observers say it is only a matter of time until our besieged neurons find new pharmaceutical allies in the form of proteins designed by nature to keep neurons alive.

–Marcia Barinaga

Some Neurotrophic Factors Moving Toward Clinical Trials

Factor	Diseases	Companies developing
Nerve growth factor (NGF)	Peripheral neuropathy Alzheimer's disease Spinal-cord injury	Genentech Synergen/Syntex
Brain-derived neurotrophic factor (BDNF)	Amyotrophic lateral sclerosis (ALS) Parkinson's disease	Amgen/Regeneron
Neurotrophin-3 (NT-3)	Peripheral neuropathy	Genentech Amgen/Regeneron Takeda
Ciliary neurotrophic factor (CNTF)	ALS Motor neuron diseases	Regeneron Synergen
Glial cell-line derived neurotrophic factor (GDNF)	Parkinson's disease	Synergen
Insulin-like growth factor I (IGF-I)	ALS Peripheral neuropathy Motor neuron diseases Post-polio syndrome	Cephalon

Additional Readings

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84.3

KAPPA OPIOIDS AND VASOPRESSIN (AVP)
J.L. Browning*, T.D. Turner, M.A. Widmayer and D.S. Baskin Dept. of Neurosurgery, VAMC Houston and Baylor College of Medicine, Houston, TX 77030.

AVP may be involved in exacerbation of cerebral injury by enhancing development of edema. Animal studies have demonstrated that central administration of AVP increases water content of normal (Rosenberg '90) and injured (Reeder '86) brain that can be reversed by AVP antagonists. Administration of kappa opioid agonists also reduces post-injury edema (Silvia '87). We have begun to investigate the reduction of AVP release as a mechanism of kappa opioid neuroprotection. Water loaded animals received i.p. injections of saline, 1 or 10 mg/kg U50488 (U50), 10μg/kg AVP, 10 μg/kg dDAVP or 10μg/kg of the V_2 antagonist [d(CH_2)$_5$, D-Ile^2, Ile^4]-AVP. In cats, compared to saline (19±19 ml per 6 hr), U50 dose dependently increased (54±18 and 124±30) urine output (UO) and plasma sodium levels (pNa), whereas the antagonist had no effect on UO (9±7 and 18±12 respectively) or pNa. Interestingly, AVP administration resulted in increased UO (68±21), although dDAVP resulted in no UO (0±0) and decreased pNa. The antagonist had no significant effect on UO (18±12) or pNa. Rabbits demonstrated similar findings, but no treatment reached significance.

In another line of investigation, we measured AVP levels in ventricular CSF in patients with head injury (HI) and control patients undergoing shunt implantation for hydrocephalus. CSF AVP levels were elevated in HI patients (1.81±0.20 vs 1.19±0.12), and correlated with intracranial pressure. These results suggest that AVP levels are increased in HI, and may be involved in the mechanism of brain edema. Kappa opioids induce a powerful diuresis, which may be neuroprotective in cerebral injury.

84.4

EFFICACY OF INSULIN-LIKE GROWTH FACTOR (IGF-1) IN THE TREATMENT OF BEHAVIORAL AND COGNITIVE DEFICITS FOLLOWING EXPERIMENTAL BRAIN INJURY.
Tracy K. McIntosh, Lei Fan, Douglas H. Smith, Mádhu Voddi, Patricia Contreras* and Thomas A. Gennarelli, Department of Neurosurgery, University of Pennsylvania, Philadelphia, PA and Cephalon, Inc. West Chester, PA.

Both insulin and IGF-1 are biologically active in the central nervous system (CNS) and IGF-1 administration has been shown to be neuroprotective in models of cerebral ischemia and hypoxic-ischemic injury. In the present study, we examined the potential efficacy of IGF-1 administration in the treatment of experimental concussive brain injury in the rat. Male Sprague-Dawley rats (250-300g, n = 29) were anesthetized (sodium pentobarbital, 50mg/kg) and subjected to lateral fluid percussion brain injury of moderate severity (2.3-2.5 atmospheres). Beginning 15 minutes following brain injury, animals received either IGF-1 (1 mg/kg i.p., n = 15) or saline vehicle (equal volume i.p., n = 14) administered twice daily at 12 hour intervals for 14 days. One subgroup of injured animals receiving IGF-1 (n = 9) or vehicle (n = 8) were evaluated for neurologic motor function at 24h, 48h, 1 week and 2 weeks following trauma using a battery of 5 composite tests. A second subgroup receiving IGF-1 (n = 6) or vehicle (n = 6) were evaluated for post-traumatic learning ability in a Morris Water Maze paradigm. IGF-1 administration did not significantly enhance the ability of brain-injured animals to learn a visuospatial task two weeks following injury. However, IGF-1 administration significantly improved neurologic motor deficits at 1 and 2 weeks postinjury. Post-traumatic administration of IGF-1 may therefore have some therapeutic value in the treatment of experimental brain injury (Supported, in part, by NS26818 and NS08803).

84.5

RESPONSES OF CORTICAL, HIPPOCAMPAL AND BRAINSTEM NEURONS TO CONTUSION INJURY: EFFECTS OF NMDA AND AMPA/KAINATE INHIBITORS.
M.Matthews*, J.Soblosky, J.Davidson, C.McInnis, M.Fogg and M.Carey, Dept. Anatomy and Neurotrauma Res. Lab., Dept. Neurosurgery, LSU Med. Ctr., New Orleans,LA 70119

An impact injury to the rat somatomotor cortex with subsequent light and EM analysis following a survival period of 3.5 minutes revealed darkened neurons within the lesion (60.3-75.8%/total) neurons) and in a 1-2mm penumbra. Reduced concentrations (20.2-43.6%/total) occurred in the lateral and pyriform cortex. Contralateral cortex displayed a spatially similar incidence of such cells. The ipsilateral hippocampal CA1 region and polymorph layer of the dentate gyrus, PAG and pontine reticular core also displayed scattered darkened cells. Examination of animals allowed to survive 2 hrs-12 wks revealed 15.4-37.8%/total darkened neurons in ipsi- and contralateral cortices. Their incidence in hippocampus, PAG and brainstem increased through 24 hours but dwindled thereafter. C-fos expression was apparent by 3.5 minutes, widespread within 10 minutes and sustained for up to 4 hours in register with regions displaying darkened cells. MK-801 partially reduced the incidence of c-fos expression whereas GYKI 52466, an AMPA/Kainate receptor inhibitor, was minimally effective in preventing the response. A pressure wave transmitted through the brain may cause much of the initial cell degeneration.

84.6

THE ADENOSINE DEAMINASE INHIBITOR, EHNA, PROVIDES CA1 NEUROPROTECTION FROM TRAUMA, HYPOXIA AND NITRIC OXIDE
J.M. Girard*, K.L. Panizzon, J. Parsons and R.A. Wallis, Dept. of Neurology, UCLA, Los Angeles, CA 90024 and Sepulveda VAMC, Sepulveda, CA 91343.

Extracellular increases in adenosine concentrations during hypoxia-ischemia have been found to be neuroprotective. Erythro-(2-hydroxy-3-nonyl)adenine (EHNA) acts as an adenosine deaminase inhibitor and produces further increase of extracellular adenosine concentrations during cerebral ischemia. Mechanical trauma and secondary hypoxia-ischemia are thought to mediate neuronal injury from head trauma, and nitric oxide (NO) has been shown to play a prominent role in both forms of injury. To assess the possible neuroprotective role of adenosine in these forms of neuronal injury, we investigated the ability of EHNA to prevent injury from trauma, hypoxia, and nitric oxide using the hippocampal slice. Treatment with 20 μM EHNA begun 30 mins. before hypoxia, improved recovery of CA1 antidromic population spike (PS) after one hour from a mean 4% ± 4 to 94% ± 1 when hypoxia was continued 5 mins. beyond the disappearance of the hypoxic injury potential. Similar protection was seen with CA1 traumatic neuronal injury. When 20 μM EHNA treatment was begun within one min. after trauma, recovery of CA1 antidromic PS improved from 19% ± 1, to 94% ± 3. Additionally, 20 μM EHNA increased CA1 antidromic PS recovery from 6% ± 3 to 87% ± 4 in slices exposed to 150 μM NO for 10 mins. under conditions of hypoxia and increased glucose concentration. These findings indicate that EHNA is neuroprotective against several forms of neuronal injury which occur with head trauma, and that elevations of extracellular adenosine concentrations may be a preventive factor against the development of neuronal injury after head trauma. Supported by the VA Research Service.

R. Rieger A. Michaelis M. M. Green

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ting → germ cell (= spore). Agametes are formed
iosis (meio-a., meiospores, tetraspores, gonia) or
): Reproduction accomplished by a. is designated
ocytogony, monogenic, or monocytogenic repro-
netic reproduction, agamogenesis, or sporogony

reproduction) asexually.
- reproduction (→ gamogenesis).
4) — asexual → reproduction. The development
a single cell. A. in unicellular organisms proceeds
rent ways:
ie cell the result of which are two roughly equal
s.
h nly a small part of the cell is constricted off.
n which the → nucleus divides several times before
ito as many parts as there are nuclei present.
eproduction of some amoeba and one phase of the
oduction cycle of sporozoa. Widely differentiated
ecialized germ cells which are called → agametes
ition to the formation of a new individual. These
is the immediate result of meiosis (in Ectocarpus,
ycetes, and angiosperms) and give rise to haploid
life cycle as constant elements of the sexual cycle.
duced mitotically by haploid or diploid organisms
dividuals with the same chromosome complement

— the asexual individual, or resp. the asexual
metangia the agametes are formed (→ gamont).
29) — an apomictic population whose members
apomixis).
apomixis: the formation of seeds without sexual
ntitious embryony, diplospory, or apospory. The
mously or after pollination (→ pseudogamy). In
he pollen does not make any genetic contribution
t is necessary to initiate the growth of the ovary
rm nucleus.
ical pathways of a. are alike in bypassing both
the cell leading to the new embryo. This results
n of seeds containing embryos which are geneti-
aternal parent.
, a. replaces sexual reproduction completely
ts some seeds form by agamospermous processes
sses (*facultative a.*).
— the complete set of factors assumed to be
tion of sexual organs and expression of sexual
sex-determining. Those factors acting to produce
nated by A, those acting to produce female sex
e carried by the autosomes (or the cytoplasm).

In a diploid cell, A and G each occur twice as AAGG, in a haploid cell only once each. Thus each cell possesses the possibility of development in both directions. Which of the two directions is chosen depends on the specific → sex realizers which are designated M and F. The sex realizers act on the alternative → reaction norm evolved through the → bisexual potency and the AG-system and this is fixed in a male or female direction (→ sex determination).

agmatoploidy (*Malheiros-Gardé* 1950) — increase of chromosome number by → fragmentation of chromosomes with diffuse or multiple → centromere organization leading to → pseudopolyploidy or → pseudoaneuploidy.

agmato-pseudopolyploidy (*Battaglia* 1956) — → pseudopolyploidy.

akaryotic — without a → nucleus (→ eukaryotic; prokaryotic).

akinetic — = → acentric.

akinetoplastic — → kinetoplast.

albomaculatus (*Correns* 1904) — ref. to a → variegation or mottling ("status albomaculatus") in plants caused by genes or extrachromosomal hereditary determinants and consisting of an irregular distribution of white and green regions (white green variegation), (→ paralbomaculatus).

aleuroplast — a → leucoplast in which protein granules predominate as a storage product.

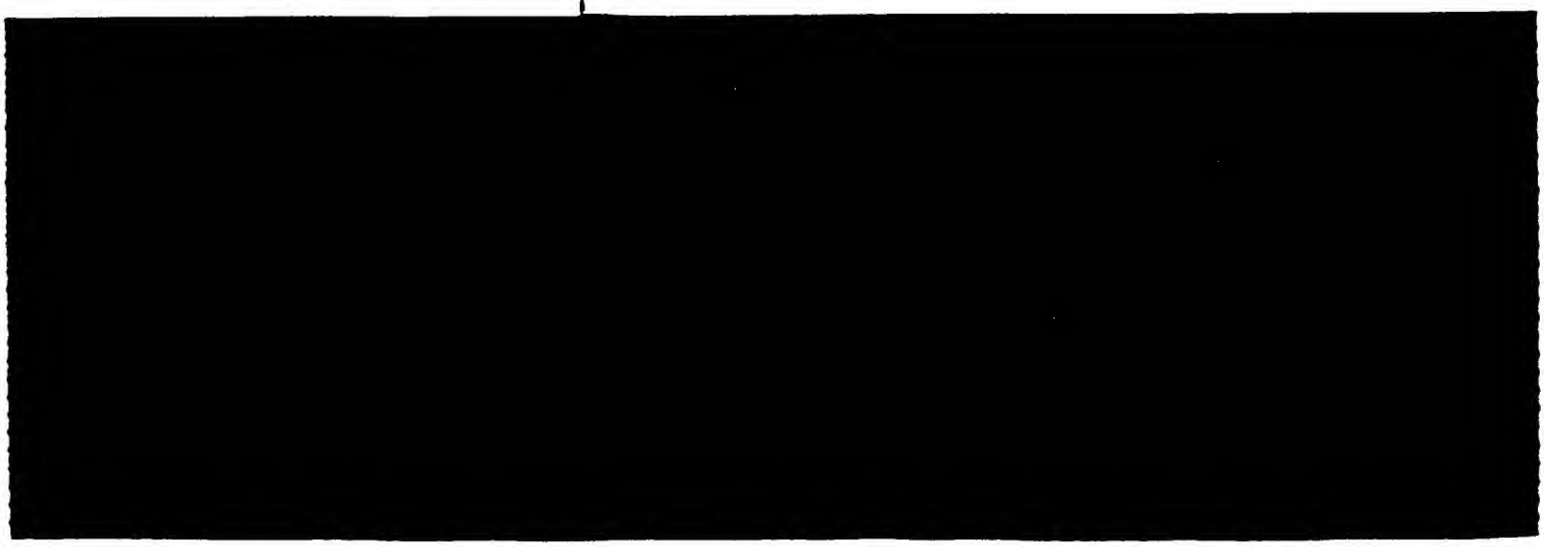
algeny (*Lederberg* 1966) — the (purposeful) altering of genes in the body cells or in germinal tissues, or the introduction of desired genes from outside (= "genetic engineering" or "genetic surgery").

alien addition line (*Leighty & Taylor* 1924; *O'Mara* 1940) — a line with an extra chromosome (monosomic addition) or an extra chromosome pair (disomic addition) from a related species (→ substitution line; alien substitution line).

alien substitution line (*Kattermann* 1938; *Unrau* et al. 1956) — a line in which an alien chromosome or a pair of alien chromosomes from a donor species replace one chromosome or a pair of chromosomes of the recipient species (→ alien addition line; substitution line). If a pair of alien chromosomes compensates for the missing chromosomes they are considered → homoeologous to the pair they replace.

allele (*Johannsen* 1909) — one of two or more alternate forms of a → gene occupying the same → locus on a particular chromosome or linkage structure and differing from other alleles of that locus at one or more → mutational sites whose number per gene is between 10^2 and 10^3. Members of a set of alleles are mutually exclusive → genetic markers and arise by → gene mutation. Their activity is concerned with the same biochemical and developmental process. A haploid organism or phase of the life cycle has a single representative of each a., a diploid two, a polyploid more than two from the total number existing in the population as a whole.

With respect to a given locus a diploid may be homo- (two identical alleles in the pair of homologous chromosomes) or heterozygous (two different alleles). If the phenotype of a heterozygous allele pair (A/a) resembles that of the homozygous pair (A/A), A is said to be dominant, a recessive. Any new allele may be characterized by its genotypic effect when it is homozygous and by its phenotypic effect when combined in heterozygotes



with a sister allele, that is, they are known by their actions and interactions (→ gene interaction). On this basis the following classes of alleles may be distinguished:

1. **amorphs** (*Muller* 1932): inactive alleles which act as → genetic blocks to normal biosynthesis. In the broadest sense an amorph may be an a. that is inoperative, one that fails to produce a measurable effect, or even the absence (→ deletion) of a gene.

2. **hypomorphs** (*Muller* 1932): alleles that function imperfectly in comparison with → wild-type alleles; sometimes known as → leaky genes. They are prevalent among induced gene mutations.

3. **hypermorphs** (*Muller* 1932): alleles that produce an excess amount of a product (the antithesis of hypomorphs), whereby excess is defined in terms of the wild-type alleles.

4. **antimorphs** (*Muller* 1932): alleles with an action opposite to that of the wild-type; this type is rare and poorly defined.

5. **neomorphs** (*Muller* 1932): alleles whose action differs qualitatively from that of the wild-type allele. Heterozygotes of neomorphs commonly display the allelic products of both alleles.

6. **isoalleles** (*Stern & Schaeffer* 1943): alleles producing only very slight differences in phenotypic expression making their identification bound to the use of special methods.

The individual alleles of a group consisting of more than two are called a **series of "multiple alleles"** (*Morgan* 1914). A diploid individual or phase of the life cycle may be homozygous for any one allele or heterozygous for a combination of any two. → Segregation in meiosis results in gametes with only a single a. The dominance relations among multiple alleles vary from one group to another. For some groups of alleles every homo- and heterozygous → genotype produces a different → phenotype. In others, the alleles may be arranged in a descending series in which every a. is dominant over all alleles below it. The number of genotypes in a series of multiple alleles is $1/2[n \cdot 1 (n + 1)]$ where n is the number of alleles of the group (for four alleles: $1/2 (4 \cdot 5) = 10$). Multiple alleles of a gene affect similar parts or processes in an organism and are indicated by attaching distinguishing letters or numbers as superscripts to the base symbol (→ genetic nomenclature).

Different alleles may produce detectable effects upon the variability at any stage in the life history of an individual and may modify the x-pected phenotypic segregation ratio so that certain classes of offspring are in excess, in reduced frequency, or are totally absent (→ lethals).

Two recessive mutations of independent origin are allelic if the heterozygote of m/m' has a mutant phenotype. If among the products of meiosis in heterozygotes — but not in homozygotes — an occasional revertant or an a. different from the two original ones arises, the two alleles are different. Different alleles are called "nonidentical" (*Demerec* 1956) (in contrast to identical alleles) when recombination has been obtained between them. The "nonidentity" refers to the fact that each represents a mutation at a different mutational site. Another term is "heteroallelic" (*Roman* 1956) for combinations of two alleles of different mutational origin which yield